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GRANT NO: **DAMD17-93-J-3010**

TITLE: **A New Class of Membrane Active Drugs for the Treatment of Breast Cancer Cells Transplanted into Athymic Mice**

PRINCIPAL INVESTIGATOR: **Kirpal S. Gulliya, Ph.D.**

CONTRACTING ORGANIZATION: **Baylor Research Institute**
3812 Elm Street
P.O. Box 710699
Dallas, TX 75226

REPORT DATE: **December 7, 1994**

TYPE OF REPORT: **Final Report**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	7 December 1994	Final (12/1/92 - 11/30/94)

4. TITLE AND SUBTITLE
A New Class of Membrane Active Drugs for the Treatment of Breast Cancer Cells Transplanted into Athymic Mice

5. FUNDING NUMBERS
Grant No.
DAMD17-93-J-3010

6. AUTHOR(S)
Kirpal S. Gulliya, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor Research Institute
3812 Elm Street
P.O. Box 710699
Dallas, Texas 75226

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Materiel Command
Fort Detrick
Frederick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The main objective of this study was to determine the effect of preactivated merocyanine 540 (pMC540) and merodantoin on the growth of established human MCF-7 breast cancer xenografts and study the toxicity, biodistribution and plasma clearance of merodantoin. Treatment of established tumors with pMC540 and merodantoin resulted in a 74% and 84% inhibition of tumor growth, respectively. Under the conditions of estradiol deprivation, this inhibition was reduced to 42% and 25%, respectively. Combined treatment with either pMC540 or merodantoin plus tamoxifen did not produce a significant enhancement of tumor growth inhibition. The LD_{50} and LD_{90} of merodantoin were determined to be 1042 mg/kg and 1250 mg/kg, respectively. The plasma half-life of merodantoin was 25 hours. Organ specific accumulation of merodantoin was not observed and majority (>70%) of the drug was eliminated via urine and feces. Both pMC540 and merodantoin were found to mediate their cytotoxic effects via initial interaction with topoisomerase II. There is a correlation between DNA topoisomerase II activity and drug-sensitive (MCF-7) and -insensitive (MDA-MB-231) breast cancer cell lines. Breast cancer cells resistant to pMC540 and merodantoin were found to contain undetectable or very low levels of topoisomerase II.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer, Growth suppression, Merodantoin, pMC540, Tamoxifen, Lab Animals, Mice, RAD VI	37
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Gurbax Singh Gallya 12/15/94

PI - Signature Date

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Annual Report
DAMD 17-93-J-3010

In 1993, over 750,000 new cases of breast cancer were diagnosed world wide and it is estimated that towards the end of this century over 1 million new cases of breast cancer will be diagnosed annually (1). Breast cancer affects one in nine American women. Breast cancer as a disease or its treatment has evoked stronger emotions than perhaps any other disease. The reasons for this can be found both in our culture in general and in medicine in particular. Depending upon the culture and context, the breast is the symbol of motherhood, nourishment, security and represents beauty and femininity. Breast cancer has a long natural history which may provide a misleading estimate of the long term outcome. In the middle of the 19th century, the breast cancer patients typically sought attention from their physician for ulcerated lesions with painful axillary involvement of the locally advanced disease. There was no effective local or systemic therapy. Surgery generally resulted in prompt return of the disease. Later, radical mastectomy introduced by Willy Meyer (2) and W.S. Halsted(3), was an important therapeutic advance. The use of their techniques is based on the theory of cancer spread model which states that cancer starts locally and spreads via the lymphatics to the regional lymph nodes and then other distant sites. Later, in 1948, McWhirter introduced postoperative radiation therapy with simple mastectomy rather than radical mastectomy (4). Like most novel approaches, this procedure, too, met with considerable resistance at the time. At present, breast cancer is being treated with one or more combinations of several modalities; e.g., surgery, radiation therapy, anti-hormonal therapy and chemotherapy. Hormonal therapy of breast cancer is designed to decrease plasma concentration of different steroid hormones or to antagonize the biological effects of these trophic substances directly at the level of tumor cells which are involved in the growth regulation of breast cancer cells (5-11). A number of chemotherapeutic agents are used in combination with and without the support of growth factors for the treatment of disseminated breast carcinoma. The addition of other cytotoxic drugs to the combination has not led to higher response frequencies (12-14).

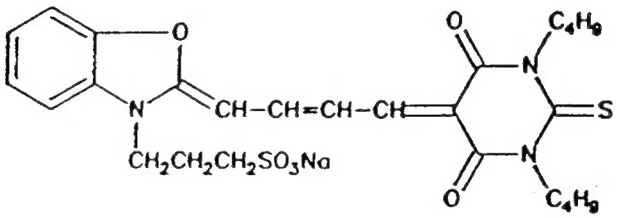
An important aspect of current breast cancer management is the early detection of tumors by mammographic screening. This strategy has provided important information regarding the natural history of the disease. However, even among women in whom earliest detectable tumor is effectively treated locally, a significant number of these patients develop clinically evident distant disease, presumably which exist as micro-metastases at the time of initial treatment. Approximately 25% of the patients with Stage I tumors (< 2 cm without axillary lymph node metastasis) and 75% of the patients with Stage II tumors (> 2 cm), develop distant metastasis despite local and regional therapy (15).

Breast cancer is unique in being sensitive to a wide variety of chemotherapeutic (and hormonal) agents. Thus the current treatments for breast cancer involves the use of many endocrine manipulations, or the use of antitumor agents principally the alkylating agents Cytoxan but also thioTEPA, mitomycin C, and L-PAM; the topoisomerase II/intercalators, doxorubicin and mitoxantrone; the antimetabolites 5-fluorouracil and methotrexate; and the vinca alkaloids vincristine and vinblastine. However, these agents by themselves or in combination have not produces entirely satisfactory results. To maximize the intensity with which these agents can be employed, these agents have been used with hematopoetic growth factors or autotransplantation or with different schedule of administration. Even so most investigators have limited expectations for these standard drugs. They believe that entirely new active agents are needed. Thus a vigorous drug discovery program has produced new clinically promising agents such as Taxol, Taxotere, which act as tubulin superstabilizer and Navelbine which acts as tubulin destabilizer; Anthrapyrazoles CI 941, a DNA intercalator; Edaterxate an antifolate compound; and Topotecan and CPT-11 which mediate their effect via inhibition of topoisomerase I. The objective response rates for Taxol and doxorubicin have been 40% to 50% in good risk stage IV patients and it is not too unrealistic to aim to achieve very high levels of partial and complete responses particularly with continued efforts towards finding compounds with improved efficacy (12, 16). Thus the need for new agents for breast cancer treatment is widely recognized.

Photodynamic therapy (PDT) is an alternative modality for the clinical management of a variety of tumors. Although photodynamic activity of certain natural products was used therapeutically in ancient times, the modern interest in photosensitizing agents for cancer therapy started with the use of hematoporphyrin derivative by Lipson, et al.,(17) in 1966 and then later by Dougherty, et al, (18) in 1975. Dougherty's group partially purified hematoporphyrin derivative and tentatively identified the photoactive constituent of

hemoglobin as dihematoporphyrin ether (DHE) which they found to be relatively more effective in cancer treatment (19-21). During the past decade, there has been increasing interest in the use of photoactive substances for cancer therapy, a subject of recent reviews (22-24). Many research groups are evaluating PDT by using a wide range of sensitizers for the treatment of various neoplasms.

Given a choice between surgery, radiation therapy and chemotherapy, the first choice of treatment for a great majority of patients has been chemotherapy. This being the case, we must ask: is it even possible that any single chemical agent will ever be an effective treatment for cancer ? The answer is clearly 'yes' which is evident by the fact that at present vigorous efforts of drug discovery programs of the National Cancer Institute and pharmaceutical industry are fertile and chemotherapy armamentarium for breast cancer is expected to double providing more than 30 active cytotoxic agents each with high expectations. In finding a new therapeutic agent one of the requirement is that the new agent has to be better than the those already available. Therefore, it is important to ask perhaps again: what are the are most desirable properties one must strive for in a chemotherapeutic agent ? For ideal cancer therapy, among the two most important requirements are 1) treatment must be effective in destroying the cancer and 2) treatment must be sparing of the host. Thus, the obvious things to look for is the specificity or selectivity of the new chemotherapeutic agent. The chances of a compound being specific for a target e.g. cancer cells, increases if the structure of the compound is special or unique. One such compound merocyanine 540, whose structure is shown below, displays special structural properties.

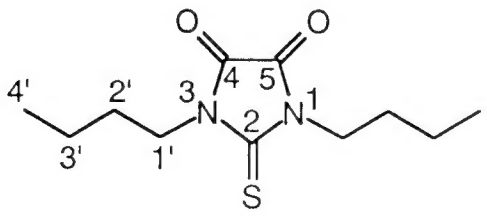


Merocyanine 540

One remarkable property of merocyanine 540 is that in the presence of serum proteins it binds to leukemia , lymphoma and certain other types of other malignant cells but bind very little if any to normal cells. Tumor cells associated with merocyanine 540 are

killed upon illumination (25, 26). During early investigation where the emphasis was on the mechanism of action as to how merocyanine kills tumor cells, it became apparent that toxicity produced was more due to in situ bleached material (*termed preactivated merocyanine 540) rather then the purported singlet oxygen generated at excited states (27-35). Other investigators have now shown that pre-illumination of merocyanine 540 produces photoproducts which could play a role in cytotoxicity (36). Similarly, it has been shown that pre-illumination of many porphyrins in simple solution results in the formation of products that do not absorb light appreciably (37). We have previously shown that preactivated photofrin II (i.e. photoproducts) is effective in killing breast cancer cells (30). Formation of stable and unstable photoproducts from other photoactive compounds has been reported (38, 39). A major advantage of using photoproducts derived from the process of preactivation is that unlike conventional photodynamic therapy, these compounds can be used for the treatment of 1) tumors too small to be visible to the naked eye, 2) metastatic diseases, and 3) viral infections. In addition, the long shelf life (months) of these photoproducts argues against the involvement of singlet oxygen generated at excited states, which is believed to be the sole cytotoxic agent in photodynamic therapy. It is important to note that 1) the involvement of singlet oxygen in the actual photodynamic therapy process is plausible and in certain cases, highly likely but completely unproven, and that 2) a correlation does not always exist between the observed cytotoxicity and the yield of singlet oxygen (26). Therefore, involvement of photoproducts in observed cytotoxicity cannot be overlooked. Two United States and several foreign patents on the process of preactivation have been issued.

Preactivated merocyanine 540 is a crude mixture of photoproduct compounds. Later some of the compounds from purposely bleached merocyanine were isolated, characterized and chemically synthesized (40). During the testing of these chemically synthesized pure compounds, it was clear that merodantoin (structure shown below) produced remarkable cytotoxicity towards malignant cells particularly breast cancer cells and it was easily tolerated in vivo (41, 42).



Merodantoin

Purpose:

The purpose of this study has been to determine the efficacy of novel photoproducts in pMC540 against human breast cancer cells transplanted into athymic mice. In the first year of this project we demonstrated that both pMC540 and merodantoin were effective in controlling the growth of freshly transplanted solid breast tumors. Results of this study are now published (41). The second (final) year (1994) of this project was devoted for the determination of the effects of pMC540 and merodantoin against established human breast tumors. The biodistribution, plasma clearance and toxicity of merodantoin was also determined. Identical experiments were also proposed for merocil, another active isolate obtained from pMC540. However, chemical synthesis of merocil turned out to be extremely difficult due unstable nature of synthesized merocil. Therefore in lieu of proposed experiments with merocil, the following experiments were carried out. During the course of this research, it was observed that in contrast to human breast cancer cell line MCF-7, the human breast cancer MDA-MB-231 cell line was resistant to the cytotoxic action of pMC540 and merodantoin. Because in vitro cytotoxic activity of pMC540 and merodantoin appeared to be dependent on the initial interaction with topoisomerase II, the role of topoisomerase II in the sensitivity and insensitivity of breast cancer cells was investigated. Results obtained suggest that insensitivity of MDA-MB-231 breast cancer cells correlates with the low levels of topoisomerase II in these cells.

Materials and Methods

Chemicals:

Time release tamoxifen pellets (5 or 10 mg/pellet; 60 day release) and 17β-estradiol (1.7 mg/pellet, 60-day release) were obtained from Innovative Res. of America (Ohio). Merocyanine 540 was purchased from Kodak-Eastman Fine Chemicals (Rochester, NY). Merodantoin (N,N'-dibutyl-2-thio-4,5-imidazolidion) was originally isolated and purified from pMC540 and synthesized in our laboratories. Stock concentrations of merodantoin were adjusted to 50 mg/mL in dimethyl sulfoxide.

Preactivation of MC540:

Merocyanine 540 (1 mg/mL) in 70% aqueous ethanol was preactivated by exposure to a bank of fluorescent lamps (GE cool white, 40 W) for 18 hours (27). After

preactivation, ethanol was removed by rotaevaporation, and the final concentration was adjusted to 50 mg/mL in 2.5% ethanol:PBS, pH 7.2.

Cells and Animals:

Human breast cancer cell line MCF-7 was purchased from American Type Culture Collection (Rockville, MD), and monolayer cultures were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 0.25 mmol/L L-glutamine, 25 mmol/L HEPES, and 25 μg/mL gentamicin sulfate (Gibco, Grand Island, NY) and kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Athymic BALB/c nude-nu mice (6–8 weeks old, 18–22 g each) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) and maintained in a germ-free environment. The mice had free access to sterile food and water.

In Vivo Tumor Growth and Hormone Treatments:

The growth of estrogen-dependent MCF-7 cells in nude mice is dependent on exogenous estrogens(43, 44). Three days prior to the injection of MCF-7 cells into nude mice, 17β-estradiol in 60-day time-release pellet form was transplanted subcutaneously using a sterile trocar needle. Estrogen pellets were not removed for the duration of the experiments. On day 4, human MCF-7 breast adenocarcinoma cells ($1x10^7$) were injected under the skin, between the panniculus musculosus and fascia. Solid tumors appearing within 45 days were transplanted serially into estradiol pellet–bearing animals. From this point onwards, a group of separately maintained nude mice served as a source of solid breast tumors for all subsequent experiments.

The effects of pMC540 (250 mg/kg) and merodantoin (75 mg/kg), was determined in mice bearing established breast tumor xenografts. For these experiments, tumors (2 x 2 mm) were implanted in estradiol pellet bearing mice and allowed to grow to an approximate size of 5 x 5 mm over a period of 10 to 12 days. Next, the treatment of the control (vehicle only) and experimental groups with pMC540, tamoxifen, and merodantoin was initiated. Tamoxifen (5 mg/pellet) was implanted subcutaneously by using a sterile trochar needle. Solid tumors using MDA-MB-435 human breast cancer cells were developed and used in an identical manner except estradiol or tamoxifen were not used. pMC540 and merodantoin were injected intramuscularly in alternate hind limbs on alternate days for 40 days. In experiments mimicking estradiol deprived conditions, the estradiol pellets were surgically removed 3 days prior to the initiation of drug treatment.

In situ measurements of tumor xenografts were made using Vernier calipers, and L x W x H were recorded. At the end of the experiments, tumor xenografts were excised, and their wet weight and size were determined. The tumor volumes were measured by the water displacement method using a pyknometer. For the calculation of tumor area and tumor volume, the following formulas were used, respectively:

$$\text{Tumor area} = {}^{l}/_{2} \times {}^{w}/_{2} \times \pi;^{(45)} \text{ ; Tumor volume (V)} = Pw-(Pt-T)D^{(46)}$$

where l = tumor length, w = tumor weight; Pw = weight of the pyknometer filled with water, Pt = weight of the pyknometer filled with water plus tumor, T = tumor weight, and D = the density coefficient of pure water.

Biodistribution:

For biodistribution studies,[^{3}H-merodantoin was injected i.p. into BALB/c mice. Three separate sets of experiments (n =3 animals/set) were done for each time point. The total dose given was 90 μCi/mouse in a final volume of 50 μl. Injected mice were kept in individual metabolic cages. Urine and fecal samples were collected from the "catch pans" of the cages. Vital organs were removed, homogenized separately, solubilized in Scintigest tissue solubilizer (Fisher Scientific) for 24-48h, and decolorized with 30% hydrogen peroxide to reduce quenching (47). Samples were then dissolved in toluene-based scintillation cocktail (containing 2,5-diphenyloxazole and 2,2'-*p*-phenylene-bis[5-phenyloxazole]). The radioactivity in each dissolved whole organ from each animal was measured separately using a model LS 1701 Beckman scintillation counter (Beckman Instruments, Nuclear Systems Operations, Fullerton, Calif.).

Plasma Half-Life:

The plasma half-life of p-[^{3}H]-merodantoin was determined following an i.p. injection of 90 μCi in a final volume of 50 μl into BALB/c mice. For each time point, three separate sets of experiments were done (n-4/set). Blood samples were obtained from the heart at various time points. Heparin was used as an anticoagulant. These samples were diluted with phosphate-buffered saline (pH = 7.4) to reduce color quenching by plasma (the quenching caused by phosphate-buffered saline was less than that produced by plasma alone). The diluted blood samples were centrifuged at 1,500 *g* for 10 min to pellet the cellular component. An aliquot from the supernatant was counted in 10 ml

scintillation cocktail. Counts per minute (CPM) were adjusted for the total volume and converted into units of decay per minute (DPM). The plasma half-life of p-[^{3}H]-merodantoin was calculated by nonlinear regression analysis.

In Vivo Toxicity:

Merodantoin was evaluated for its toxicity in three animal species. Three separate sets of experiments were performed using BALB/c mice (n = 10 per set per dose) for determinations of the 90% toxic dose (LD_{90}) and the 50% toxic dose (LD_{50}) of merodantoin. The dose range used was 300-1250 mg/kg. In repeat dose experiments mice were given i.p. injections of merodantoin and followed for a period of 30 days. A control group (n = 10) of animals were injected with an equal volume of the drug solvent (DMSO).

Chemicals used in the studies related to topoisomerase II:

Form 1 kDNA, pRYG DNA substrate for Topo II, Topo II monoclonal antibody, Topo II 170 kD marker and m-AMSA were purchased from TopoGen, Inc. (Columbus, OH). Plasmid pBR322 DNA, DMEM, L-15 media, and FBS were obtained from GIBCO (Gaithersburg, MD). ATP, proteinase K, and PMSF were obtained from Sigma (St. Louis, MO). Protoblot II AP system with stabilized substrate was obtained from Promega Corp. (Madison, WI). Human MDR1-specific cDNA probe was obtained from ATCC (Rockville, MD). MAb C219 was purchased from Signet Laboratories, Inc. (Dedham, MA).

Cell Lines and Determination of Sensitivity of Growing Cells to TopoisomeraseDependent compounds:

MCF-7 (breast carcinoma, human) and MDA-MB-231 (breast adenocarcinoma, human) were obtained from ATCC (Rockville, MD); both were maintained in continuous culture in DMEM and L-15 medium supplemented with 10% FBS. Both cell lines grew as monolayer cultures at 37°C in a humid atmosphere with 5% CO_2.

Cells were seeded in 25 cm^2 tissue culture flasks to give approximately 400 colonies/flask and were allowed to attach overnight. Cells were treated with the desired concentrations of test compounds. After a 24- to 48-h treatment, cells were washed with drug-free medium to remove the compounds, and 5 ml of fresh medium was added to each flask. After 14 days of incubation at 37°C, growth medium was removed, and 1 ml of 0.5

% toluidine blue dye was added. After one min the dye was removed and the flasks were washed with PBS. The total colonies in each flask were manually counted.

Southern Blotting, Northern Blotting, and Western Blotting of the P-glycoprotein:

Molecular biology procedures were carried out according to standard procedure (48). MDR1 gene amplification and mRNA expression of the MDR1 gene were verified on DNA and RNA blots by hybridization to human MDR1-specific cDNA probe. Genomic DNA blots, RNA blots, and hybridization conditions were as described (49, 50). Blots were washed to a stringency of 0.1 x standard saline citrate (1 x is 150 mM NaCl:15 mM sodium citrate, pH 7.0) at 65°C for 30 min. RNA was isolated using the acid guanidium thiocyanate-phenol chloroform extraction method (51). Over expression of the P-glycoprotein was tested with the monoclonal antibody MAb C219, which is directed against P-glycoprotein (52).

Preparation of Whole Cell Lysates:

Breast cancer cells were removed from tissue culture flasks by scraping, pelleted by centrifugation at 1500 x g for 3 min at 4°C, and washed once in PBS. The pellet was resuspended in solubilization buffer (PBS with 2% SDS, 1mM phenylmethylsulfonyl fluoride, 1mM benzamide, 10 μg/ml soybean trypsin inhibitor and 25 μg/ml leupeptin) at 2.5 x 10^7 cells/ml. The cell suspension was sonicated (Heat Systems-Ultrasonics, Inc. Cell Disrupter, Model W-225 R), and stored at –20°C. Protein concentrations were determined with the Bradford (53) protein assay (Bio-Rad, Richmond, CA), with bovine serum albumin (BSA) as a standard.

Preparation of Cellular and Nuclear Enzyme Extracts:

Cells were extracted as described previously (54). All procedures were performed on ice. Cells were harvested, and a washed cell pellet was obtained as described for preparation of whole cell lysates. The pellet was resuspended at 1 x 10^7 cells/ml in extraction buffer A (10 mM Tris-HCl [pH 7.5], 25 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM PMSF) and allowed to stand at 0°C for 15 min. Lysis was achieved by pipetting the suspension 50–60 times with a commercial P 200 Pipetman. The molarity of NaCl in cell lysates was adjusted to 0.5 M by addition of an equal volume of extraction buffer B (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 M NaCl, 1 mM dithiothreitol, 1 mM PMSF) and gently homogenized. After extraction for 2 h at 4°C the mixture was

centrifuged at 16000 x g for 20 min at 4°C. Protein concentrations were determined as described earlier. The enzyme solution was diluted with an equal volume of 87% glycerol and stored at –20°C.

Nuclear extracts were prepared by modification of a published report (55). Exponentially growing cells were collected by centrifugation and washed in ice cold buffer NB (NB consists of 2 mM K_2HPO_4, 5 mM $MgCl_2$, 150 mM NaCl, 1 mM EGTA, and 0.1 mM dithiothreitol, pH adjusted to 6.5). The washed cells were resuspended in NB, and 9 ml of NB, supplemented with 0.3 % Triton-x-100 and 1 mM PMSF was added slowly down the side of the tube. The cell suspension was mixed by rotation for 5 min at 4°C, centrifuged at 1000 x g for 10 min, and the nuclear pellet washed in Triton-free NB. Nuclear protein was extracted from the nuclei for 30 min at 4°C with ice cold NB containing 0.5 M NaCl (final concentration). DNA and nuclear debris were pelleted by centrifugation at 17000 x g for 10 min and the supernatant collected. Protein concentration in the extract and storage of the enzyme solution was done as described above.

Topo II Catalytic Activity Assay:

Topo II catalytic activity was assayed using the decatenation assay (56). The standard reaction mixture for the decatenation assay was 50 mM Tris-HCl (pH 7.5), 85 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, bovine serum albumin (0.03 mg/ml), and 1 mM ATP (57). Decantenation of kDNA was carried out by incubating 5 µl nuclear or cellular extract with 2 µg of kDNA in a final volume of 25 µl standard reaction mixture for 30 min at 37°C. Reactions were terminated by the addition of 5 µl of 3% SDS, 0.3% bromophenol blue, and 30% glycerol. Samples were then electrophoresed in 1% agarose in 89 mM Tris-borate, 2 mM EDTA (pH 8.3) at 35 V for 4 h. Gels were stained with ethidium bromide (1.0 µg/ml) for 45 min and destained for 2–3 h in deionized H_2O. DNA bands were visualized by transillumination with UV and photographed.

Reaction conditions for Topo II-mediated DNA Cleavage:

Topo II-induced cleavage of DNA was assayed by the generation of form III (linearized) DNA from supercoiled (form 1) pRYG DNA in the presence and absence of drugs (58, 59). The reaction mixture for DNA cleavage was 30 mM Tris-HCl (pH 7.6), 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$, and 60 mM NaCl. Topo II-induced DNA cleavage was carried out by incubating 5 µl cellular or nuclear extract with pRYG 0.3 µg and the test compounds in a final volume of 20 µl standard reaction mixture for 30 min at 37°C. The reactions were terminated by the addition of 2 µl SDS (10%) and 2.0 µl

proteinase K (1 mg/ml), while the samples were carefully kept at 37 °C (60). Following an additional incubation for 30 min at 37 °C, 6 µl of 0.3% bromophenol blue and 30% glycerol were added. Samples were extracted with equal volume of chloroform: isoamyl alcohol (24:1), vortexed briefly, and spun in a microfuge for 5 s. Blue-colored upper phase was withdrawn and analyzed by gel electrophoresis in 1% agarose at 20 V for 16 h in a manner identical to the one used in the decatenation assay.

Topo I Catalytic Activity Assay:

Topo I activity was assayed by relaxation of supercoiled pBR322 DNA (61). The standard reaction mixture used for the Topo I activity assay was the same as described above for the Topo II assay, except ATP and Mg^{2+} were omitted. Relaxation was carried out by incubating 5 µl cellular or nuclear extract with supercoiled DNA (0.9 µg) in a final volume of 25 µl standard reaction mixture for 30 min at 37°C. Reactions were terminated by the addition of 5 µl of 3% SDS, 0.03% bromophenol blue, and 30% glycerol. Samples were then analyzed by gel electrophoresis in 1% agarose at 20 V for 16 h as described in the decatenation assay. Some stained gels (in 0.1 µg ethidium bromide/ml) were subjected to an additional 3 h of electrophoresis at 20 V in the presence of 0.1 µg ethidium bromide per milliliter to separate relaxed closed circles and nicked circles, since ethidium bromide increases the mobility of the relaxed closed circles relative to the nicked circles (62).

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting:

Whole cell lysates (150 µg total protein) from normal exponential growing breast cancer cells were size fractionated in 7.5 % polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulfate (63). Samples were dissolved in sample buffer for 5 min at room temperature before loading. Proteins from the gel were electrotransferred onto a nitrocellulose sheet as described (64) by using a Bio-Rad transfer apparatus. Blots were blocked with 1% BSA in TBST and probed with anti-170 kD mouse monoclonal antibody directed against human Topo II (TopoGen, Inc.) appropriately diluted in TBST. Blots were incubated with antibody for 2 h at room temperature. Following three 10-min washes in TBST, the blots were incubated with alkaline phosphatase-conjugated goat antimouse IgG at room temperature for 1 h. Bands were visualized after washing the blot with Tris-saline buffer and incubating with substrate for alkaline phosphatase as described by the supplier (Promega Corp., Madison, WI).

Statistical Analysis:

Results are presented as the arithmetic mean x ± SD for each control and experiment group. Differences among the x of groups were determined using Student's two-tailed *t* test (In Stat 1.12, GraphPad computer program for Macintosh), and values of $P < .05$ were considered significant.

Results:

Effect of pMC540 on established estrogen-dependent MCF-7 human breast tumor xenografts:

MCF-7 human breast tumor xenografts bearing nude mice were treated with 250 mg/kg pMC540. Treatment was started approximately three weaks after tumor implantation. Single injections of pMC540 were given on alternate days for five weeks. Results show that this dosing regimen inhibited tumor growth (*Figure 1*) rapidly and continued for the duration of the experiments. At the end of the experiment final tumor weights of treated and control animals were compared. Results show (Table 1) that in treated mice, tumors growth was eventually reduced by 75% as compared to the untreated controls. However, in the presence of 5 mg or 10 mg tamoxifen (time release pellets), this inhibition was only marginally increased to 83% and 93% respectively (*Table 1*). Weight loss or other observable changes in the general well being of the treated animals were not observed confirming previous findings that pMC540 is easily tolerated.

Effects of merodantoin on established estrogen-dependent MCF-7 human breast tumor xenografts:

Growth of established MCF-7 tumors in mice treated with merodantoin (75 mg/kg), given as a single intramuscular injection on alternate days, was prevented for the duration of the experiment (*Figure 2*). A final comparison (*Table 2*) of the excised tumors revealed that merodantoin caused an 85% reduction in tumors growth whereas a maximum inhibition of 93% was observed in animals treated with tamoxifen plus merodantoin. Tamoxifen alone at doses of 5mg and 10 mg caused an 18.5% and 89% inhibition of tumor growth. Therefore, combined effect of merodantoin and tamoxifen did not produce a significant improvement in the observed inhibition of tumor growth.

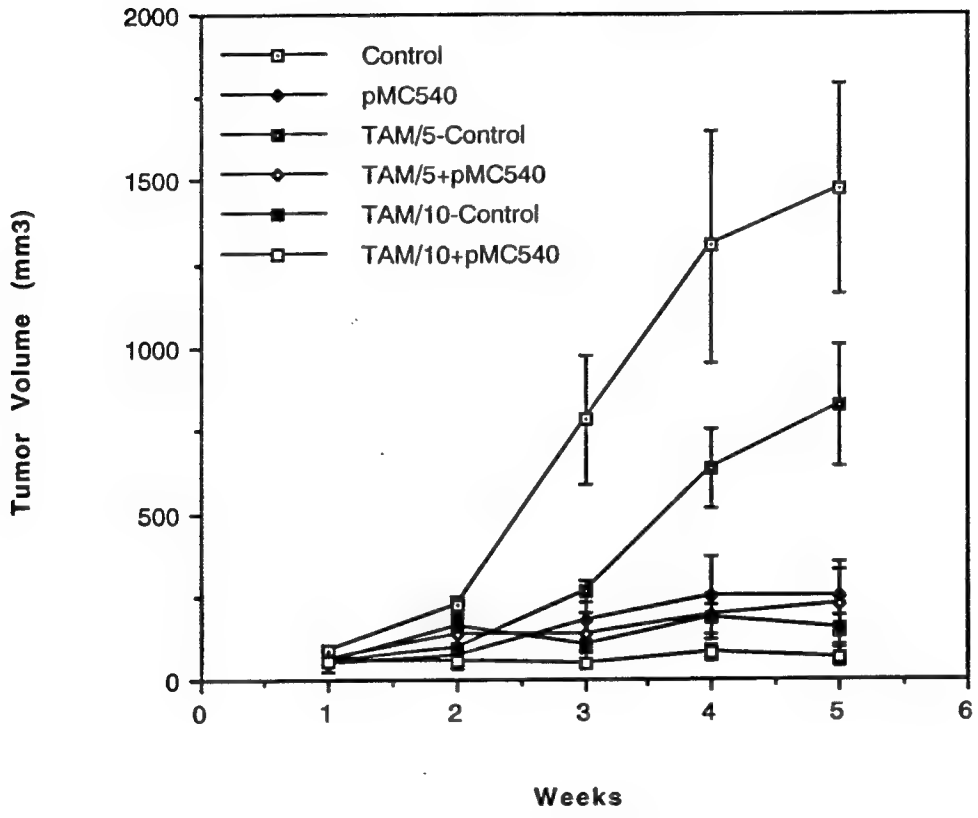


Figure 1. Growth curves (mean+ standard deviation) of established MCF-7 tumor xenografts in the continuous presence of estrogen (control) and treated with pMC540 (250 mg/kg), tamoxifen (TAM) 5mg and 10 mg pellets, and TAM plus pMC540. Alternate-day treatment of mice was initiated 10 days after tumor implantation. There were 10 mice per group.

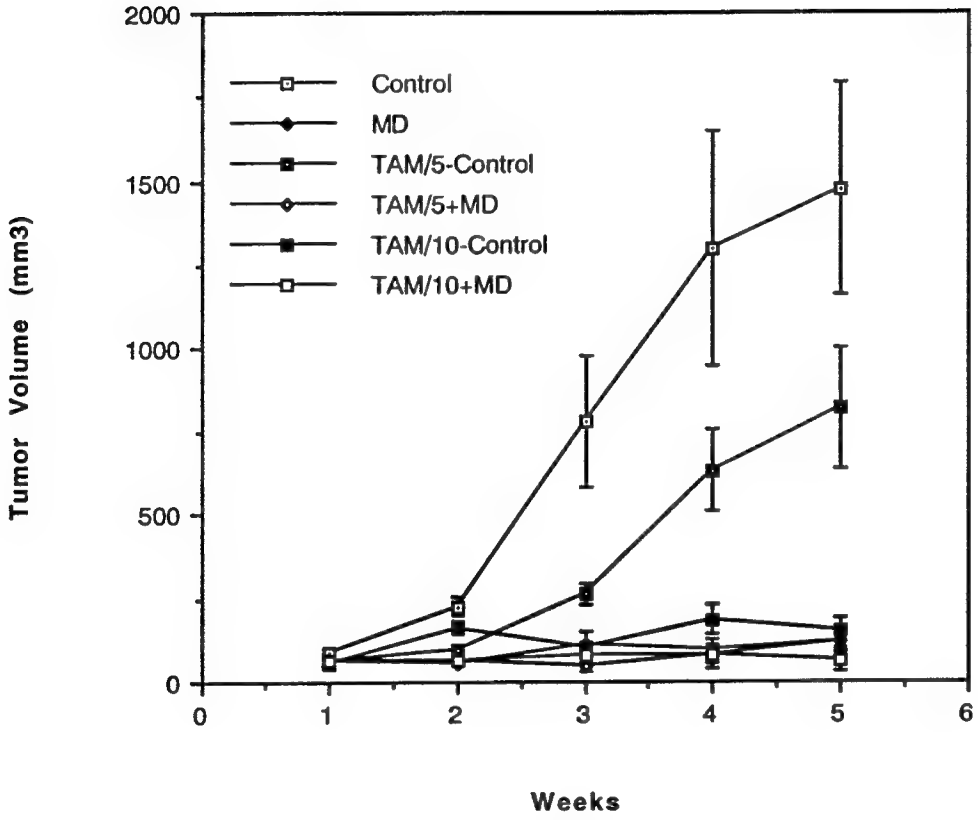


Figure 2. Growth curves (mean+ standard deviation) of established MCF-7 tumor xenografts in the continuous presence of estrogen (control) and treated with Merodantoin (MD; 75 mg/kg), tamoxifen (TAM) 5mg and 10 mg pellets, and TAM plus merodantoin (MD). Alternate-day treatment of mice was initiated 10 days after tumor implantation. There were 10 mice per group.

Table 1. Effect of pMC540 and tamoxifen on established breast tumors.

Treatment	Dose	Area (mm^2)	Weight (gm)	Vol. (mm^3)	% TI
Control	vehicle only	162.5 ± 22.9	1.283 ± 0.119	1475.8 ±314.21	
pMC540	250 mg/kg	51.7 ± 7.0	0.326 ± 0.062	240.3 ± 40.8	74.6%
TAM	5 mg	92.1 ± 13.1	1.059 ± 0.177	821.2 ± 184.8	18.5%
TAM	10 mg	33.0 ± 8.0	0.200 ± 0.045	150.2 ± 44.3	84.6%
TAM + pMC540	5 mg + 250 mg/kg	43.1 ± 14.1	0.227 ± 0.118	226.8 ± 100.1	82.5%
TAM + pMC540	10 mg + 250 mg/kg	18.7 ± 6.2	0.088 ± 0.031	67.4 ± 26.8	93.2%

Implantation tumor size approximately 2 x 2 mm^2. Tumors were allowed to grow to an approximate size of 5 x 6 mm (approximately 8–10 days). Treatment with pMC540 were given on alternate days via intramuscular injection for 30 days. Final tumor size assessment were made on day 34.

% TI indicates percent tumor growth inhibition, calculated using the formula %TGI = 100 $(1-W_t/W_C)$, where W_t and W_C are the mean weights of the treated and controlled tumors respectively.

Table 2. Effect of merodantoin and tamoxifen on established breast tumors.

Treatment	Dose	Area (mm^2)	Weight (gm)	Vol. (mm^3)	% TI
Control	vehicle only	162.5 ± 22.9	1.283 ± 0.119	1475.8 ±314.21	
MD	75 mg/kg	31.9 ± 5.2	0.191 ± 0.048	119.1 ± 29.8	84.6%
TAM	5 mg pellet	92.1 ± 13.1	1.059 ± 0.177	821.2 ± 184.8	18.5%
TAM	10 mg pellet	33.0 ± 8.0	0.200 ± 0.045	150.2 ± 44.3	84.6%
TAM + MD	5 mg + 75 mg/kg	25.3 ± 8.6	0.136 ± 0.047	115.8 ± 43.7	89%
TAM + MD	10 mg + 75 mg/kg	18.2 ± 6.8	0.086 ± 0.033	64.98 ± 3.41	93.1%

Implantation tumor size approximately 2 x 2 mm^2. Tumors were allowed to grow to an approximate size of 5 x 6 mm (approximately 8–10 days). Treatment with pMC540 were given on alternate days via intramuscular injection for 30 days. Final tumor size assessment were made on day 34. %TGI was calculated as described in legend for table 1.

Effect of pMC540 and merodantoin on estradiol deprived established MCF-7 human breast tumor xenografts:

Human MCF-7 breast tumor xenografts were transplanted into estrogen pellet bearing mice and tumors were allowed to grow. When tumors grew to an approximate size of 5 X 5 mm, the estrogen pellets were removed and an alternate day treatment with pMC540 (250 mg/kg) or merodantoin (75 mg/kg) in the presence and absence of tamoxifen was initiated. Removal of estrogen pellets caused a rapid initial regression followed by stationary phase of tumors in all groups (*Figure 3 and 4)*. None of the treatment produced a significant difference in tumor regression as compared to the untreated control. However, in the presence of tamoxifen (10 mg) both pMC540 and merodantoin caused a stimulation of tumor growth. This increased growth was more pronounced in case of pMC540 then merodantoin.

Effect of pMC540 and merodantoin on non-estrogen dependent established MDA-MB- 435 breast tumors:

Human MDA-MB 435 breast tumor xenofrafts were transplanted into athymic mice with out the exogenous estradiol support. Established tumors were treated as described above. Treatments with pMC540 or merodantoin were effective in causing a 59% inhibition of tumor growth. Treatment with merodantoin caused an initial tumor regression (34%) which lasted for two weeks followed by stationary phase in tumor growth. Since MDA-MB-435 tumors are known to metastasize to lungs, the effect of this treatment on metastasis was also investigated. Gross examination of lungs from treated group revealed that the proportion of large tumors in lungs was reduced in treated animals as compared to the untreated contro group. However, this reduction was statistically not significant (P> 0.5). These data suggest that pMC540 and merodantoin may be effective in controlling the growth of estrogen dependent and independent breast tumor growth and optimization of drug dose and injection schedule may improve upon the observed efficacy.

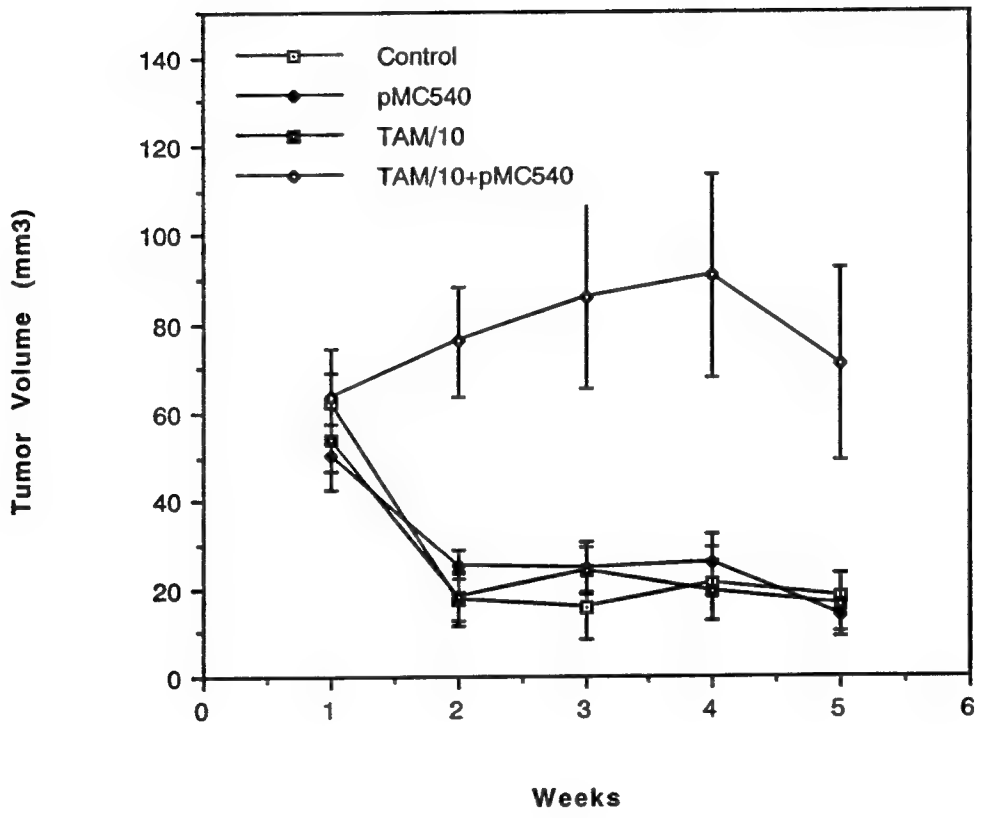


Figure 3. Growth curves (mean+ standard deviation) of established MCF-7 tumor xenografts under the estradiol deprived conditions (control) and treated with pMC540 (250 mg/kg), tamoxifen (TAM) 5mg and 10 mg pellets, and TAM plus pMC540. Alternate-day treatment of mice was initiated 10 days after tumor implantation. There were 10 mice per group.

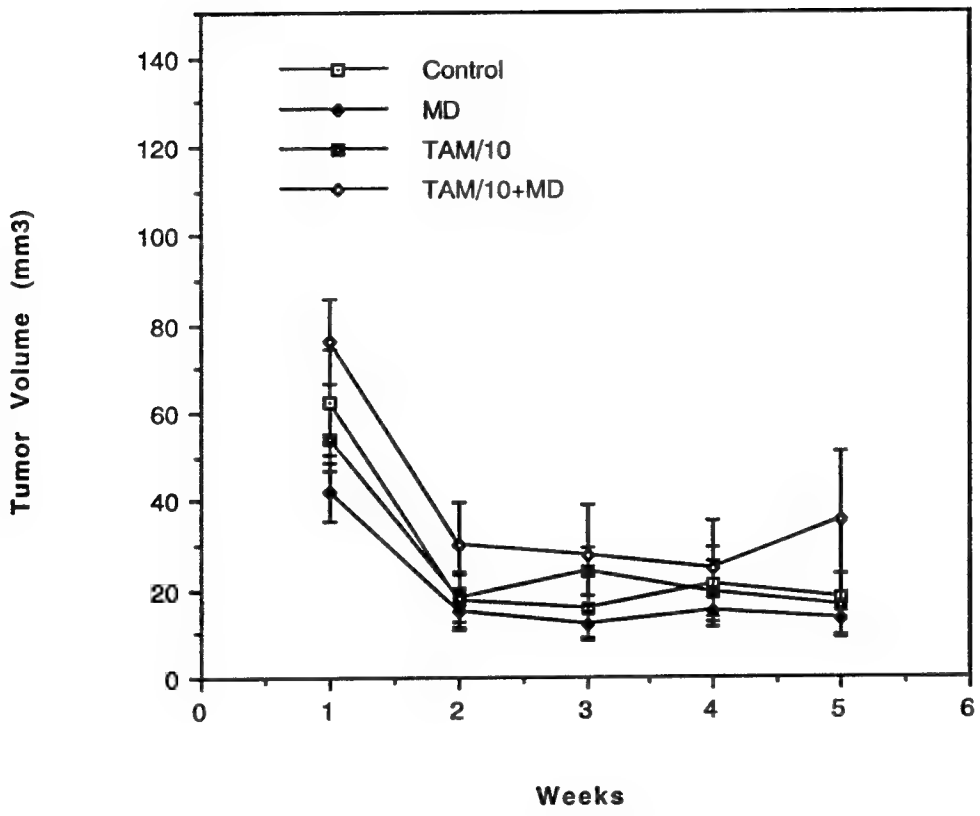


Figure 4. Growth curves (mean+ standard deviation) of established MCF-7 tumor xenografts under the estradiol deprived conditions (control) and treated with Merodantoin (MD; 75 mg/kg), tamoxifen (TAM) 5mg and 10 mg pellets, and TAM plus merodantoin (MD). Alternate-day treatment of mice was initiated 10 days after tumor implantation. There were 10 mice per group.

Table 3. Effect of pMC540 and tamoxifen on estradiol deprived established breast tumors.

Treatment	Dose	Area (mm^2)	Weight	Vol.(mm^3)	%TGI
Control	vehicle only	7.13 ± 3.27	0.024 ± 0.02	18.3 ± 10.1	
pMC540	250 mg/kg	7.04 ± 1.37	0.014 ± 0.01	13.8 ± 3.5	41.7%
TAM	10 mg	6.55 ± 2.28	0.017 ± 0.01	16.0 ± 7.1	29.1%
TAM + pMC540	10mg/250mg/kg	21.48 ± 4.93	0.107 ± 0.04	70.9 ± 21.5	

Implantation size of tumors 2 x 2 mm. Tumors were allowed to grow to an approximate size of 5 x 5 mm (approximately 8–10 days). Prior to the treatment estradiol pellets were removed. Treatment with merodantoin were given on alternate days for 30 days. Final assessment of tumor weights were made on day 33. %TGI was calculated as described in legend for table 1.

Table 4. Effect of merodantoin and tamoxifen in estradiol deprived established breast tumors.

Treatment	Dose	Area (mm^2)	Weight	Vol. (mm^3)	%TGI
Control	vehicle only	7.13 ± 3.27	0.024 ± 0.02	18.3 ± 10.1	
Merodantoin	75 mg/kg	7.30 ± 1.58	0.018 ± 0.01	13.10 ± 3.6	25%
TAM	10 mg	6.55 ± 2.28	0.017 ± 0.01	16.0 ± 7.1	29.2%
TAM + MD	10 mg/75 mg/kg	11.73 ± 4.95	0.044 ± 0.02	35.3 ± 16.0	

Implantation size of tumors 2 x 2 mm. Tumors were allowed to grow to an approximate size of 5 x 5 mm (approximately 8–10 days). Prior to the treatment estradiol pellets were removed. Treatment with merodantoin were given on alternate days for 30 days. Final assessment of tumor weights were made on day 33. %TGI was calculated as described in legend for table 1.

In vivo toxicity and biodistribution of merodandoin:

The in vivo toxicity of merodantoin was determined in BALB/c mice. Merodantoin at doses of 750 mg/kg given as single intraperitoneal injections were easily tolerated. The LD_{50} of merodantoin was 1042 mg/kg and LD_{90} was determined to be 1250 mg/kg. In repeat dose experiments (n=10) it was determined that daily single injection of merodantoin (750 mg/kg) were easily tolerated for three days, however, with continued treatment, animals began to die off on day four and a 100% mortality occured by day 20. Identical experiments were carried out using low doses of merodantoin. At a dose of 400 mg/kg (n=6), single daily injections of merodantoin were easily tolerated for six days and with continued treatment a 100% mortality occured by day 18.

An investigation of the biodistribution of ^{3}H-merodantoin shows (*Table 5* , next page) that none of the major organs displayed a preferential accumulation of radioactivity. Highest activity was observed in liver accounting for approximately 4.5% radioactivity and the remaining organs accounted for 1.5% or less radioactivity. Majority (over 70%) of the radioactivity was recovered from urine and feces by 4 hours after the injections. It is noteworthy that subsequent to 4 hour time point, the urine and feces data which amounts to 80% of radioactivity, are not reflected in the total amount of recovered activity.

Altogether, the total drug elimination accounts for >90% of the radioactivity recovered. After 24 hours, the excretion of the radioactivity via urine and feces was reduced to approximately 12%, indicating that over 70% of the radioactivity has been excreted.

The plasma half-life of ^{3}H-merodantoin was evaluated after a single injection of 90 μCi into BALB/c mice. Blood samples were collected at various time points and the activity in the plasma was determined. Data show (*Figure 4A)* that the maximal concentration of the drug in the plasma was obtained 2 hours after an i.p. injection. Results show that at 25 hours after the injection, the plasma-drug level was very close to the half-maximal drug level. The plasma half-life was calculated by nonlinear regression analysis.

Table 5. Biodistribution of ^{3}H-merodantoin

SAMPLES	2 h	4 h	6 h	8 h
LIVER	219,354 ± 17,281 (4.5)	195,849 ± 52,667 (4.1)	131,633 ± 28,444 (2.7)	88,882 ± 28,753 (1.8)
KIDNEY	58,869 ± 16,837 (1.2)	57,820 ± 9,003 (1.2)	37,153 ± 6,594 (0.8)	24,032 ± 8,641 (0.5)
SPLEEN	14,684 ± 4,666 (0.3)	15,485 ± 1,404 (0.3)	10,500 ± 3,967 (0.2)	6,431 ± 2,850 (0.13)
HEART	22,642 ± 5,185 (0.4)	19,417 ± 3,019 (0.4)	13,730 ± 3,046 (0.3)	7,649 ± 2,554 (0.16)
BRAIN	65,961 ± 17,729 (1.4)	64,685 ± 13,677 (1.3)	40,897 ± 8,841 (0.8)	31,139 ± 5,697 (0.65)
LUNGS	14,537 ± 1,560 (0.3)	19,046 ± 2,237 (0.4)	11,711 ± 3,515 (0.2)	6,795 ± 3,112 (0.14)
URINE	1,254,069 (26)	2,311,434 ± 67,922 (48)	1,817,340 ± 564,025 (38)	782,004 ± 287,520 (16.2)
FECES	164,993 (3.4)	114,323 ± 67,922 (2.4)	101,298 ± 15,126 (2.1)	194,649 ± 49,120 (4.0)

Table 5. (cont.) Biodistribution of ^{3}H-merodantoin

SAMPLES	24 h	48 h	72 h	96 h
LIVER	78,625 ± 8,115 (1.6)	45,739 ± 8,462 (0.93)	45,174 ± 14,082 (0.94)	25,527 ± 6,083 (0.53)
KIDNEY	21,698 ± 4,647 (0.45)	14,503 ± 997 (0.30)	11,323 ± 3,101 (0.23)	12,201 ± 2,179 (0.25)
SPLEEN	5,616 ± 1,084 (0.12)	3,508 ± 89 (0.07)	3,152 ± 1,064 (0.06)	3,063 ± 383 (0.063)
HEART	7,909 ± 1,247 (0.16)	5,094 ± 818 (0.11)	3,302 ± 669 (0.3)	4,215 ± 206 (0.5)
BRAIN	22,845 ± 770 (0.5)	22,001 ± 2,522 (0.5)	13,072 ± 2,993 (0.3)	21,547 ± 2,302 (0.5)
LUNG	6,151 ± 1,137 (0.13)	3,443 ± 780 (0.07)	3,326 ± 805 (0.07)	3,730 ± 713 (0.08)
URINE	274,453 (5.7)	184,501 (7.0)	-	-
FECES	323,335 ± 98,981 (6.7)	338,597 ± 143,835 (7.0)	-	-

Data represents mean values of three separate experiments consisting of 3 animals/group. The radioactivity values in urine and feces, accounting for over 50%, are not reflected in the amount of radioactivity recovered subsequent to 4-h time point. Data in parentheses is the percentage of total radioactivity injected.

Table 5. (cont.) Biodistribution of ^{3}H-merodantoin

ORGAN	7 days	14 days
LIVER	14,795 ± 1,200 (0.31)	7,300 (0.15)
KIDNEY	3,207 ± 518 (0.07)	3,835 (0.08)
SPLEEN	848 ± 183 (0.02)	959 (0.02)
HEART	1,042 ± 206 (0.022)	651 (0.013)
BRAIN	4,003 ± 551 (0.083)	1,723 (0.04)
LUNG	879 ±184 (0.02)	672 (0.014)

Data represents mean values of three separate experiments consisting of 3 animals/group. The radioactivity values in urine and feces, accounting for over 50%, are not reflected in the amount of radioactivity recovered subsequent to 4-h time point. Data in parentheses is the percentage of total radioactivity injected.

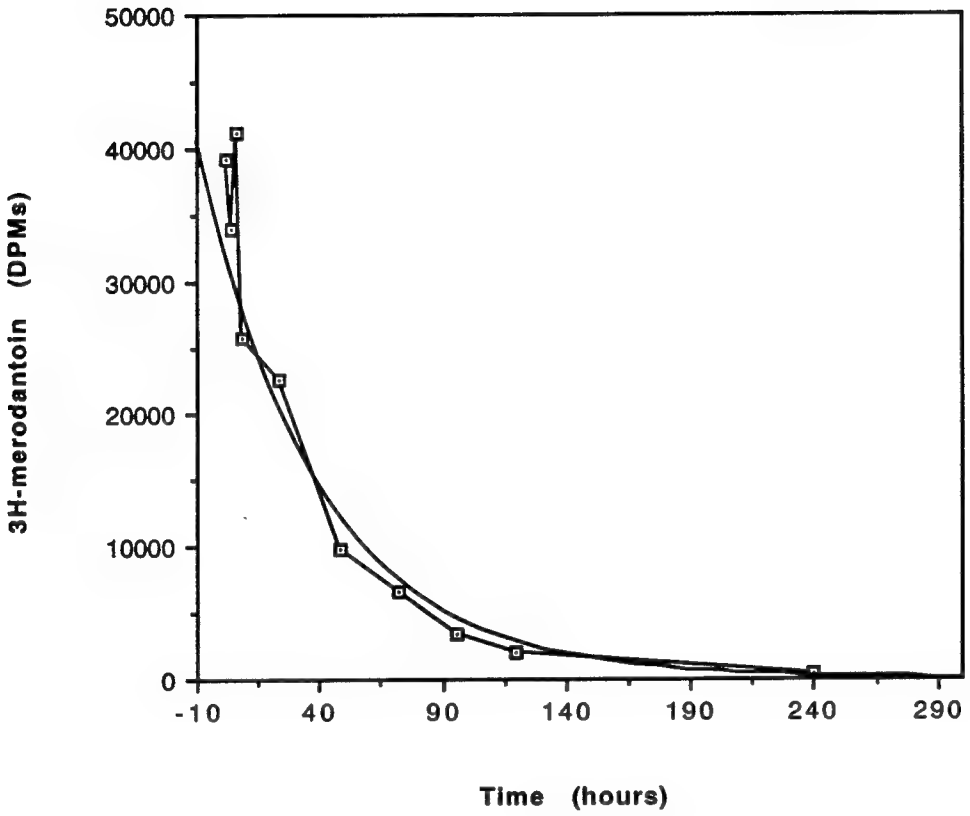


Figure 4A. Plasma clearence of ^{3}H-merodantoin was determined by an intraperitoneal injection of 90 μCi into BALB/c mice. Blood samples were collected at various time points and diluted with PBS (pH 7.4) and centrifuged at 1500 g for 10 minutes to pellet the cellular components. Radioactivity of the cell free plasma was determined by using a model LS 1701 Beckman scintillation counter.

Sensitivity to Topo II Dependent pMC540 and Merodantoin:

In clonogenic assays (*Figure 5*), treatment of MCF-7 cells with 80 μg/ml of pMC540 (140.4 μM) for 48 h caused a 99.4% inhibition of colony formation. Similarly, treatment of MCF-7 cells with 10 μg/ml of merodantoin (41.3 μM) for 48 h, caused a virtually complete inhibition of colony formation. However, an identical treatment of MDA-MB-231 cells with pMC540 caused a 38% inhibition of clonogenic growth. Treatment of MDA-MB-231 cells with merodantoin (41.3 μM) also was significantly reduced as it caused only a 33% inhibition of clonogenic growth. Higher doses of pMC540 (263.25 μM) were also ineffective and merodantoin upto a dose of 103.3 μM produced only a 58% inhibition of clonogenic growth. A longer period (72 h) of treatment of MDA-MB-231 cells with these agents was ineffective in producing an enhanced inhibition of clonogenic growth. These data suggest that MDA-MB-231 cells are significantly less susceptible to the cytotoxic action of pMC540 and merodantoin.

P-glycoprotein Expression and the Possibility of Drug Resistance in MCF-7 and MDA-MB-231:

To determine whether the observed insensitivity of MDA-MB-231 cells to pMC540 and merodantoin is related to the expression of MDR phenotype, both MCF-7 and MDA-MB-231 cells were analyzed for mdr1 gene amplification, mRNA over expression of the mdr1 gene, and P-glycoprotein expression. Southern blotting did not reveal mdr1 gene amplification in both cell lines. Over expression of mRNA of the mdr1 gene was not detected, and western blot using C219 monoclonal antibody was also negative for both cell lines (results not shown). These results suggest that the involvement of multidrug resistance is unlikely, and the observed resistance to pMC540 and merodantoin cannot be explained by MDR involvement.

Topo II and Topo I Catalytic Activity:

In previous studies, in vitro analysis revealed that the activity of pMC540 and merodantoin is dependent on Topo II (42). Therefore, catalytic activity of Topo II obtained from cellular and nuclear extracts of breast cancer cells was determined by decatenation of the kDNA network (56). We determined Topo II activity in crude nuclear extract as well as in crude cellular extracts from MCF-7 and MDA-MB-231 in several different experiments, after the extract protein concentrations were adjusted to equivalence. In both cell lines, the total cellular protein concentration ranged from 1.35–1.41 mg/10^7 cells. Results show that Topo II activities were two- to threefold lower in crude nuclear extracts from MDA-MB-231 than in extracts from MCF-7, as could be seen by comparing band intensities of the

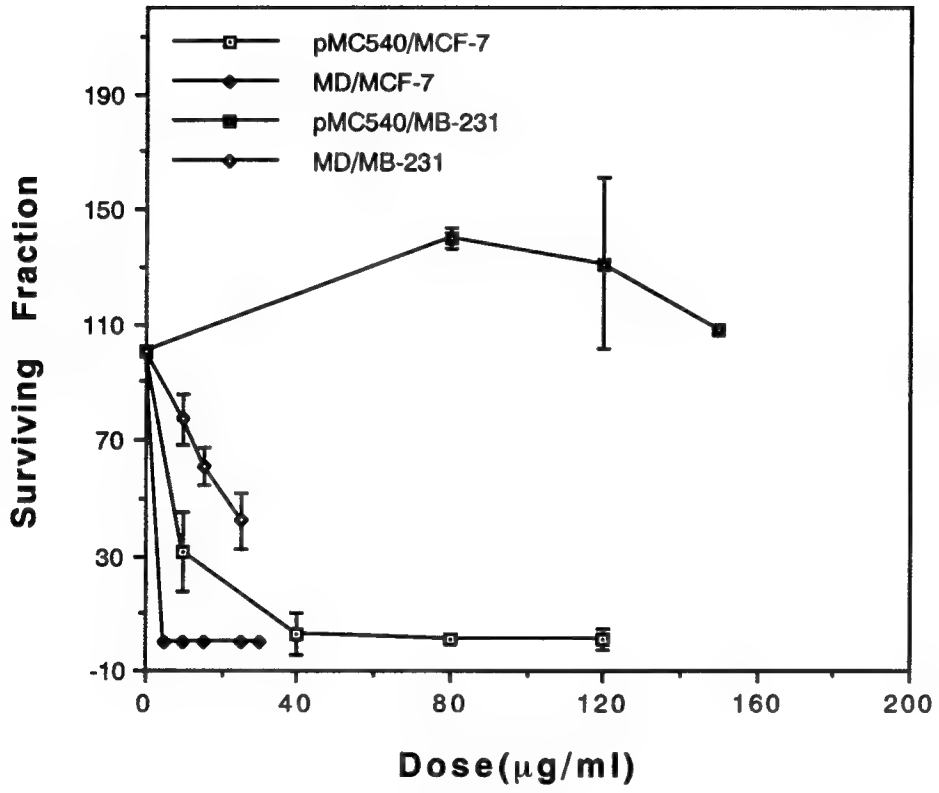


Figure 5. Clonogenic growth of human breast cancer MCF-7 and MDA-MB231 cells treated with different doses of pMC540 and merodantoin (MD). Cells were treated for 48 hours and then drugs were removed and allowed to grow in drug free growth medium. After 14 days of incubation at 37^{o} C, the growth medium was removed and toluidine blue stained colonies were counted. Results (mean±SD) of three separate experiments are shown.

minicircles in the serial dilutions (*Figure 6)*. The bands near the top of the gel were products of incompletely decatenated kDNA (56). In crude cellular extracts from MDA-MB-231, Topo II activities were undetectable, in the case of MCF-7, minicircles were detectable (data not shown). In the absence of ATP, decatenation of kDNA was undetectable (results not shown). To exclude the possibility that the decrease in Topo II activity in extracts from MDA-MB-231 was an unspecific phenomenon, Topo I catalytic activities were determined in the same extracts. Topo I activities in extracts from MCF-7 and MDA-MB-231 were compared by determining the highest dilution factor that was needed for the ATP-independent relaxation of all supercoiled pBR322 DNA (61). The Topo I activities were almost identical in nuclear extracts (*Figure 7)* and cellular extracts (results not shown) from both cell lines. The relaxation of supercoiled pBR322 was completely due to Topo I activity and not endonuclease activity in these extracts, because all of the newly formed relaxed circles appeared to be closed circles (as determined by gel electrophoresis) in the presence of ethidium bromide (results not shown).

pMC540 and Merodantoin-induced DNA Cleavage Activity:

Whether the reduced Topo II activity in nuclear extracts from MDA-MB-231 has an effect on the drug-induced formation of the cleavage complex was studied in an in vitro DNA cleavage assay using supercoiled pRYG and nuclear extract (59, 60). Both pMC540 and merodantoin stimulated the Topo II-mediated DNA cleavage in the presence of ATP and nuclear extracts, as could be seen (*Figure 8)* by the formation of linearized pRYG DNA (form III) after SDS proteinase K treatment. Increased linearized pRYG DNA was detectable in extracts from MCF-7 cells in the presence of pMC540 and merodantoin as compared to extracts from MDA-MB-231. This is probably due to decreased Topo II activity in MDA-MB-231. The addition of high salt (0.5 M NaCl) after pre-incubation with pMC540 and merodantoin (*Figure 9)* reduced the DNA cleavage significantly in nuclear extracts from MCF-7 as well as MDA-MB-231.

Inhibition of Topo II Catalytic Activity in MCF-7 and MDA-MB-231 in the Presence of m-AMSA:

Because the Topo II catalytic activity was reduced two- to threefold and the formation of the cleavable complex was also reduced in nuclear extracts from MDA-MB-231, we wondered if m-AMSA treatment would inhibit the Topo II activity more effectively in nuclear extracts from MDA-MB-231 than from MCF-7. The Topo II activity in the presence of m-AMSA was monitored by the decatenation assay (56). Results show (*Figure 10)* that m-AMSA completely inhibited the decatenation activity in extracts from MDA-MB-

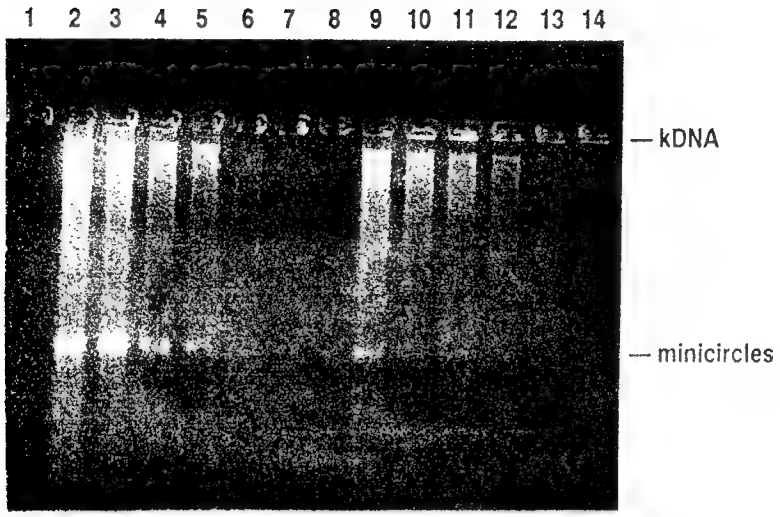


Figure 6. Topoisomerase II activity in nuclear extracts from MCF-7 and MDA-MB-231. Topoisomerase II activity was monitored by the decatenation assay. Reaction mixture (25 μl) containing 2.0 μg of kDNA and various dilutions of nuclear extracts from MCF-7 (lanes 2–7) or MDA-MB-231 (lanes 9–14) and blank (lane 8) were incubated for 30 min at 37° C and analyzed as described under "Materials and Methods." The amounts of extract protein added were: control, no extract protein (lane 1), 2.5 μg (lanes 2 and 9), 1.25 μg (lanes 3 and 10), 0.625 μg (lanes 4 and 11), 0.313 μg (lanes 5 and 12), 0.156 μg (lanes 6 and 13), 0.078 μg (lanes 7 and 14).

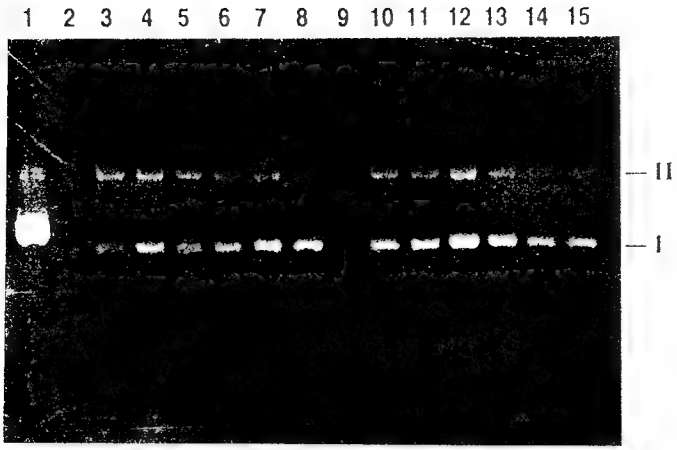


Figure 7. Topoisomerase I activity in nuclear extracts from MCF-7 and MDA-MB-231. Reaction mixture (25 µl) containing 0.9 µg of supercoiled pBR322 and various dilutions of nuclear extracts from MCF-7 (lanes 3–8) or MDA-MB-231 (lanes 10–15) and blank (lanes 2 and 9) were incubated for 30 min at 37° C and analyzed. The amounts of extract protein added were control, no extract protein (lane 1), 1 µg (lanes 3 and 10), 0.5 µg (lanes 4 and 11),) 0. 25 µg (lanes 5 and 12), 0.125 µg (lanes 6 and 13), 0.0625 µg (lanes 7 and 14), 0.0313 µg (lanes 8 and 15).

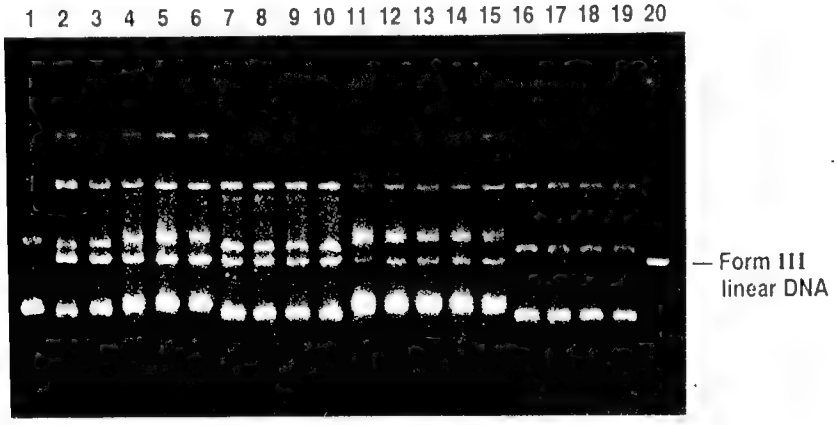


Figure 8. pMC540 and merodantoin (MD) stimulated cleavage of pRYG DNA in the presence of nuclear extracts from MCF-7 and MDA-MB-231. Various concentrations of pMC540 and MD were added to the reaction mixture (20 µl) containing 0.3 µg of supercoiled pRYG and then 2.5 µg of nuclear extract protein from MCF-7 (lanes 2–10) or MDA-MB-231 (lane 11–19) was added. After 30 min. at 37°C, the reaction was terminated with SDS, proteinase K was added and DNA were sample prepared. Lane 1, control, no drug, no extract protein; lanes 2 and 11, no drug; lanes 3–6, 35.1, 70.2, 140.4 and 210.6 µM pMC540; lanes 7–10, 20.65, 41.3, 61.95, and 103.25 µM merodantoin; lanes 12–15 and lanes 16–19 same as lanes 3–6 and lanes 7–10 respectively. Lane 20, pRYG linear DNA marker.

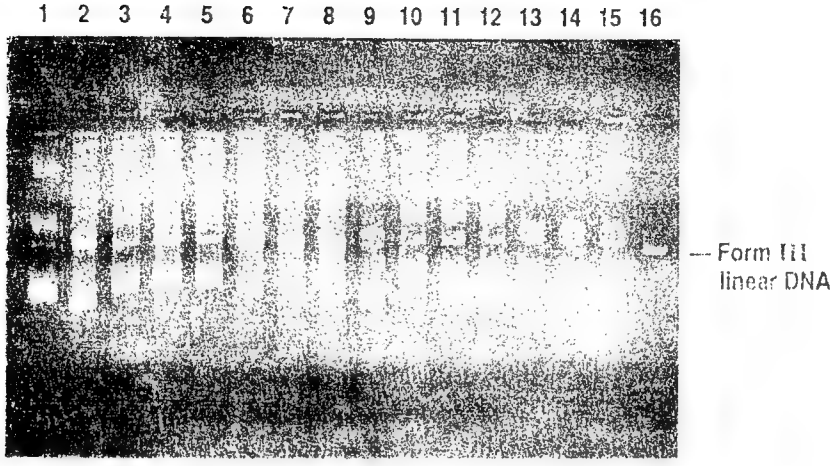


Figure 9. Reversal of pMC540 and merodantoin induced DNA cleavage by high-salt treatment. The reaction mixture (20 μl) containing 0.3 μg of supercoiled pRYG and 2.5 μg of nuclear extract protein from MCF-7 (lanes 2–8) or MDA-MB-231 (lanes 9–15) was incubated at 37° C for 30 min. in the presence of 210.6 μM pMC540 and 103.25 μM merodantoin (MD). Sodium chloride (5 M) was then added to the reaction mixture (0.5 M NaCl final). Termination of the reaction with SDS at various times after the second incubation, proteinase K treatment and DNA sample preparations were done as described under "Materials and Methods."Lane 1, control, no drug, no extract protein; lanes 2 and 9, control, no drug; lanes 3–5, pMC540; lanes 6–8 merodantoin, reactions were terminated at 0, 15 and 30 minutes after the addition of NaCl; lanes 10–12, and lanes 13–15, same as lanes 3–5 and lanes 6–8 respectively. Lane 16, pRYG linear DNA marker.

231 at a concentration of 10 μM) and in extracts from MCF-7 (at a concentration of 40 μM).

Western Blot Analysis of Topo II:

The amount of 170 kD form of Topo II enzyme in nuclear extract and whole cell lysates of the three breast cancer cell lines (T47D, MDA-MB-231 and MCF-7) was determined by Western blot analysis (*Figure 11)*. Transfer of proteins from the gel to the membrane (MATERIALS AND METHODS) was virtually complete as judged by the absence of Coomassie stainable material in the gel after transfer (data not shown). Total Topo II (170 kD) level was monitored in whole cell lysates (*Figure 11)* and nuclear extracts, which indicates that the Topo II present in the nuclear extract is representative of the Topo II present in the whole cell. Further evidence was provided by immunoblot analysis of extracted nuclei and cytosolic fractions (data not shown) which revealed no detectable Topo II (170 kD) in the cytosolic fractions and only a very low level in extracted nuclei. A similar comparison of Topo II levels (170 kD only) in cellular fractions from CHO cells showed that the Topo II present in nuclear extract is representative of the Topo II present in the whole cell (65). The amount of 170 kD enzyme is higher in MCF-7 and T47D cell lines, whereas MDA-MB-231 revealed no detectable Topo II in the whole cell lysates. (Both MCF-7 and T47D breast cancer cell lines are susceptible to pMC540 and merodantoin cytotoxicity.)

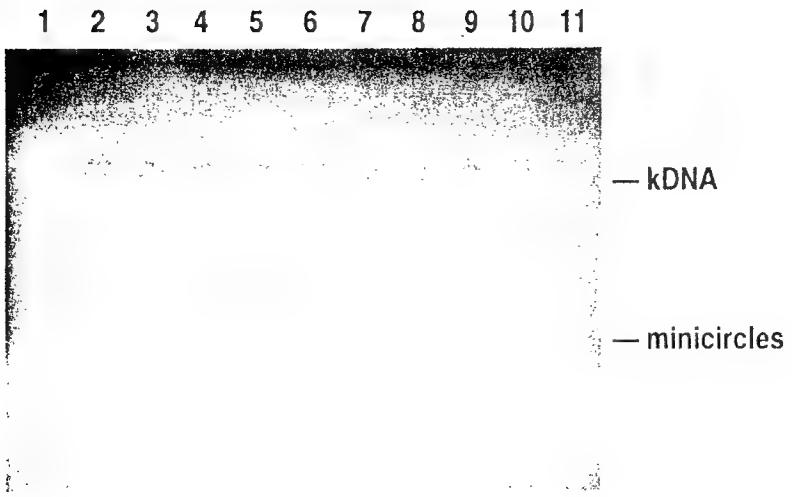


Figure 10. Inhibition of the topoisomerase II activity in nuclear extract of MCF-7 and MDA-MB-231 by m-AMSA. Topoisomerase II activity was monitored by the decatenation assay. First various concentrations of *m*-AMSA were added to the reaction mixture (25 µl) containing 2.0 µg of kDNA and then 2.0 µg of nuclear extract protein from MCF-7 (lanes 2–6) or MDA-MB-231 (lanes 7–11) was added. After 30 min. at 37° C the reaction was terminated and analyzed as described under "Materials and Methods." Lane 1, control, no drug, no extract protein; lane 2 and 7, control, no drug; lanes 3 and 8, 5 µM m-AMSA; lanes 4 and 9, 10 µM m-AMSA; lanes 5 and 10, 20 µM m-AMSA; lanes 6 and 11, 40 µM m-AMSA.

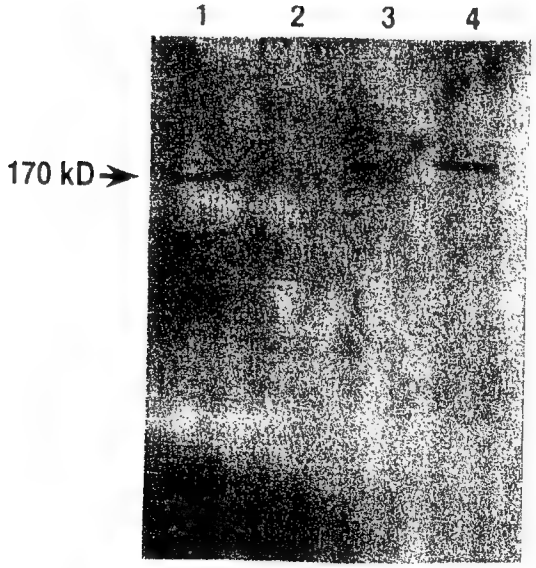


Figure 11. Western blot analysis of 170 kD topoisomerase II in whole cell lysates (150µg total protein/lane from normal exponentially growing breast cancer cell lines); lane 1, 170 kD molecular weight marker; lane 2, MDA-MB-231; lane 3, T47 D; lane 4, MCF-7. Western blotting was performed as described in "Materials and Methods." Triplicate experiments gave similar results.

Conclusions:

Data presented clearly demonstrates that both pMC540 and merodantoin are effective in inhibiting the growth of established human MCF-7 breast tumor xenografts. Merodantoin , a chemically synthesized isolate from pMC540 was slightly more effective then pMC540. The toxicity data demonstrates that these compounds are easily tolerated. For example, merodantoin was easily tolerated up to a dose of 750 mg/kg. Merodantoin did not present any specific association with any of the major organs and appears to clear primarily via urine and feces. The half life of plasma clearence was noted to be 25 hours. It is note worthy that in in vivo studies reported here, only 75 mg/kg dose administered on alternate days was used. Future studies are planned for the optimization of drug dose and schedule of drug administration to obtain a virtually complete elimination of breast tumors. In addition, we have established that these compounds mediate their cytotoxic effects via initial interaction with topoisomerase II. Breast cancer cells that are resistant to these compounds were found to contain undetectable or very low levels of topoisomerase II. Thus it appears that initial interaction with topoisomerase II is an important step in the cytotoxicity mediated by these compounds. This finding, at least in part, also explains the lack of toxicity towards normal cells and tissues which are known to contain relatively low levels of topoisomerase II as compared to the malignant cells. In a previous study, we have demonstrated that such interaction (involving topoisomerase II) eventually leads to the induction of apoptosis. Thus, under the auspices of The Department of Army, further development of these novel compounds has been advanced bringing them a step closer to potential clinical applications.

Problems:

The chemical synthesis of merocil turned out to be virtually impossible as the synthesized product was unstable. In lieu of this work, studies of the role of topoisomerase II in the cytotoxicity mediated by pMC540 and merodantoin were carried out. These studies have provided very important insights towards enhancing our understanding of the mechanism of action of these compounds. A manuscript on this subject has been submitted for publication.

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Kirpal S. Gulliya, Ph.D.
Principal Investigator

APPENDIX

Reprint:

Gulliya, K.S., Sharma, R.K., Matthews, J.L., Benniston, A.C., Harriman, A. and Nemunaitis, J.J.: In vitro and in vivo growth suppression of MCF-7 human breast cancer by novel photoproducts and tamoxifen. *Cancer* **74(6)**:1725-1732.

In Vitro and In Vivo Growth Suppression of MCF-7 Human Breast Cancer by Novel Photoproducts and Tamoxifen

Kirpal S. Gulliya, Ph.D., Radha K. Sharma, Ph.D.,* James L. Matthews, Ph.D.,* Andrew C. Benniston, Ph.D.,† Anthony Harriman, Ph.D.,† and John J. Nemunaitis, M.D.‡*

Background. **Preactivation is a novel photochemical method for the production of chemotherapeutic compounds that exert their biologic effects independent of light. The compounds that are produced, preactivated merocyanine 540 (pMC540) and merodantoin, are cytotoxic to cultured human breast cancer cells but are only minimally cytotoxic toward normal cells. Their effects against breast cancer have not been studied in vivo.**

Methods. **Estrogen-stimulated human MCF-7 breast adenocarcinoma cells were grown as solid tumors in athymic carrier mice. Animals bearing defined sizes of subcutaneously transplanted solid breast tumors received injections of pMC540 (250 mg/kg) with or without concurrent treatment with tamoxifen. Growth inhibitory effects of merodantoin (N,N'-dibutyl-2-thio-4,5-imidazolidion) on the breast tumor growth were determined.**

Results. **Direct injection of established tumors with eight doses of pMC540 (250 mg/kg) administered on alternate days resulted in significant tumor regression ($P = 0.002$). In three of seven animals, palpable tumors could not be detected after this treatment (16 days). Treatment through intramuscular injections (20 doses) with pMC540 (250 mg/kg) also caused a significant suppression of tumor area ($P = 0.004$; $P = 0.0882$; $P = 0.0903$) and a marginally significant suppression of tumor weight and volume, respectively. Combined treatment with tamoxifen and pMC540 (100 mg/kg) caused a 67% suppression of breast tumor growth. Treatment with 20 doses of merodantoin (75 mg/kg) suppressed the growth of breast tumors by 98%.**

Conclusion. **To the authors' knowledge, these results show for the first time that photochemically generated novel compounds in pMC540 alone and in combination with tamoxifen are effective in suppressing in vivo growth of xenografted human MCF-7 breast tumors. *Cancer* 1994; 74:1725–32.**

Key words: merodantoin, merocyanine 540, tamoxifen, breast cancer, growth suppression.

In 1992, breast carcinoma was diagnosed in 182,000 women in the United States; mortality attributed to breast cancer occurred in 46,000 women.[1] Less than 20% of patients with Stage IV disease are alive after 5 years of diagnosis.[2] Although much progress has been made in the early detection of breast tumors and surgical treatment, endocrine therapy and standard cytotoxic chemotherapy have not altered survival of patients with advanced disease.[3] Results of recent studies exploring dose-intense chemotherapy followed by administration of cytokines, bone marrow, and/or peripheral blood stem cells suggest improved disease free survival intervals, but mortality and morbidity associated with pancytopenia, mucositis, and other regimen-related toxicities are significant and costly.[4–6]

There is an urgent need to develop more effective agents or methods for treatment of advanced breast cancer. Tumor cytotoxic compounds derived from the novel process of preactivation are toxic to certain types of tumor cells but sparing of normal cells and tissues.[7–12] Preactivation is a process in which a photoactive compound is illuminated under specific conditions before its use in biologic systems. The singlet oxygen

From the *Baylor Research Institute, Baylor University Medical Center, Dallas; the †Center for Fast Kinetics Research, University of Texas at Austin, Austin; and the ‡Texas Oncology Physicians' Association, Dallas, Texas.

Supported by Grant DAMD17-93-J-3010 from the Department of the Army.

The authors thank Professor Burchard Franck, Organisch-Chemisches Institut, Universitat Munster, Germany, for providing purified merodantoin and Ms. Lauren Arnold, Baylor Research Institute, for technical assistance.

Address for reprints: Kirpal S. Gulliya, Ph.D., Baylor Research Institute, 3812 Elm Street, Dallas, TX 75226.

Received March 4, 1994; revision received May 24, 1994; accepted May 24, 1994.

thus produced attacks the dye molecule itself, resulting in the formation of heretofore unknown compounds.[13] New compounds thus produced exert their biologic effects independent of light and can be used for the systemic treatment of malignancy. The mechanism of biologic action of these compounds is not entirely clear, but it appears that long-lived hydroperoxides and/or superoxides induced by this compound may play a significant role in the observed cytotoxicity.[11] Although the process of preactivation is applicable to many chromophore-containing compounds, several preactivation related studies have focused on merocyanine 540 (5-[3-sulfopropyl 2(3H)benzoxazolylidine)-2-butenylidine]-1,2-dibutyl-2-thiobarbituric acid), an amphipathic dye.[7–13] In preliminary studies, it was observed that photoproducts derived from preactivation of merocyanine 540 (pMC540) are cytotoxic to cultured human breast cancer cells. Photoproducts such as merocil, meroxazole, and merodantoin have been isolated and characterized.[14] However, the effects of these compounds on the growth of MCF-7 breast tumors transplanted into athymic mice have not been studied. In the current investigation, we report that pMC540 alone and in combination with tamoxifen as well as merodantoin, a chemically synthesized compound originally isolated from pMC540, suppressed the growth of xenografted breast tumors.

Materials and Methods

Chemicals

Time-release tamoxifen pellets (5 mg/pellet; 60-day release) and 17β-estradiol (1.7 mg/pellet, 60-day release) were obtained from Innovative Research of America (Toledo, OH). Merocyanine 540 was purchased from Eastman Fine Chemicals, Eastman Kodak Co., (Rochester, NY). Merodantoin (N,N'-dibutyl-2-thio-4,5-imidazolidion) was originally isolated and purified from pMC540 and synthesized in our laboratories.[14] Stock concentrations of merodantoin were adjusted to 50 mg/ml in dimethyl sulfoxide.

Preactivation of MC540

Merocyanine 540 (1 mg/ml) in 70% aqueous ethanol was preactivated by exposure to a bank of fluorescent lamps (GE Cool White, 40 W, Cleveland, OH) for 18 hours.[4] After preactivation, ethanol was removed by rotaevaporation, and the final concentration was adjusted to 50 mg/ml in 2.5% ethanol:phosphate buffered saline, pH 7.2.

Cells and Animals

Human breast cancer cell line MCF-7 was purchased from American Type Culture Collection (Rockville, MD), and monolayer cultures were maintained in minimal essential medium supplemented with 10% fetal bovine serum, 0.25 mmol/l L-glutamine, 25 mmol/l HEPES (N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulphonic acid]), and 25 μg/ml gentamicin sulfate (Gibco, Grand Island, NY) and kept at 37°C in a humidified atmosphere of 5% carbon dioxide in air. Athymic BALB/c nude-nu mice (6–8 weeks old, 18–22 g each) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), and maintained in a germfree environment. The mice had free access to sterile food and water.

Cell Cytotoxicity Assay

Monolayer cultures of MCF-7 cells were trypsinized (trypsin–ethylenediamine tetraacetic acid 0.05%), washed three times, resuspended in complete growth medium (5×10^4 cells/ml), and seeded (100 μl/well) into 96 well plates. After overnight incubation at 37°C in a humidified atmosphere of 5% carbon dioxide in air, they were treated with different concentrations of pMC540 and merodantoin. After the indicated incubation periods, the cell survival was determined by a tetrazolium-based assay.[15] Vehicle controls were used in all experiments.

Clonogenic Assay

Cytotoxicity also was assessed by determining the surviving number of drug-treated colonies compared with control (vehicle only) cultures in clonogenic assay. To assay for the inhibition of clonogenic growth, MCF-7 cells (5×10^5 cells/ml) were treated with pMC540 (40 μg/ml and 80 g/ml) for 24–48 hours. After the incubation period, cells were plated in clonogenic growth medium containing 1 ml of 0.8% methylcellulose (Methocel A4M, Dow Chemical Co., Midland, MI), 20% fetal bovine serum, 0.6 mM 2-mercaptoethanol, 100 U penicillin, and 100 μg streptomycin. To prevent the attachment of cells to the plate, a layer of 3% methylcellulose was placed in the bottom of the wells. Alternatively, MCF-7 cells were cloned in a monolayer cloning assay in which single cells (1000 per flask in 5 ml) were plated in 25 cm^2 flasks. After overnight incubation, the cells were treated with pMC540 and merodantoin for 24 and 48 hours, washed, and replaced with fresh growth medium. Colonies consisting of 50 or more cells were manually counted after 7–10 days of growth at 37°C in a 5%

carbon dioxide humidified atmosphere. Latent growth was monitored for 15 days. There was no significant difference in the percent inhibition of MCF-7 clonogenic growth by these methods.

In Vivo Tumor Growth and Hormone Treatments

The growth of estrogen-dependent MCF-7 cells in nude mice is dependent on exogenous estrogens.[16,17] Three days before the injection of MCF-7 cells into nude mice, 17β-estradiol in 60-day time-release pellet form was transplanted subcutaneously using a sterile trochar needle. Estrogen pellets were not removed for the duration of the experiments. On day 4, human MCF-7 breast adenocarcinoma cells (1×10^7) were injected under the skin, between the panniculus musculosus and fascia. Solid tumors appearing within 45 days were transplanted serially into estradiol pellet-bearing animals. From this point on, a group of separately maintained nude mice served as a source of solid breast tumors for all subsequent experiments.

In the first set of experiments, the growth inhibitory effects of pMC540 (250 mg/kg) injected directly into the breast tumors were determined. When the implanted tumor (5 × 5 mm) was 7 × 6 mm in size, animals were randomized, and their treatment through intratumor injections was initiated. Control animals received vehicle only.

In the second set of experiments, the effect of pMC540 and one of its chemically synthesized isolates, merodantoin, was determined. For these experiments, the implantation size of the tumor was 2 × 2 mm; the treatment of the control (vehicle only) and experimental groups with pMC540, tamoxifen, and merodantoin was initiated 24 hours after tumor implantation. Tamoxifen (5 mg/pellet) was implanted subcutaneously by using a sterile trochar needle. pMC540 and merodantoin were injected intramuscularly in alternate hind limbs on alternate days for 40 days.

In situ measurements of tumor xenografts were made using Vernier calipers, and L × W × H were recorded. At the end of the experiments, tumor xenografts were excised, and their wet weight and size were determined. The tumor volumes were measured by the water displacement method using a pyknometer. For the calculation of tumor area and tumor volume, the following formulas were used, respectively:

$$\text{Tumor area} = \frac{1}{2} \times \frac{w}{2} \times \pi^{18}$$

$$\text{Tumor volume (V)} = Pw - (Pt - T)D^{19}$$

where l equals tumor length, w equals tumor weight, Pw equals weight of the pyknometer filled with water, Pt equals weight of the pyknometer filled with water plus tumor, T equals tumor weight, and D equals the density coefficient of pure water.

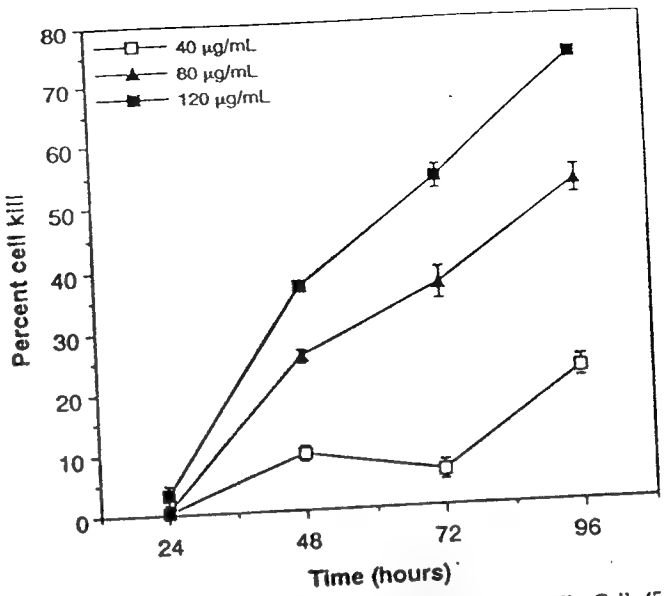

Figure 1. Dose–effect curves of pMC540 against MCF-7 cells. Cells (5×10^4 cells/ml) were treated with indicated doses of pMC540. After incubation at 37°C for 24, 48, 72, or 96 hours, the cell viability was determined by (3-[4,5-dimethyl-thiazole-2-yl]-2,5-diphenyl-tetrazoliumbromide) assay. Results (mean ± standard error of the mean) of three separate experiments performed in quadruplicate are shown.

Statistical Analysis

Results are presented as the arithmetic mean plus or minus standard deviation for each control and experiment group. Differences among the mean of groups were determined using the Student's two-tailed *t* test (Biostatistics Program Version 1.12, GraphPad computer program for Macintosh, San Diego, CA), and values of $P < 0.05$ were considered significant.

Results

Cell Cytotoxicity

In a set of in vitro experiments, breast cancer cells (MCF-7) were treated with different doses of pMC540 and merodantoin. The dose-response curves are shown in Figures 1 and 2. A maximum cell kill of 73% was observed at a dose of 120 µg/ml pMC540 after 96 hours of incubation. However, for merodantoin (Fig. 2), the cytotoxic effects also were dose dependent but more

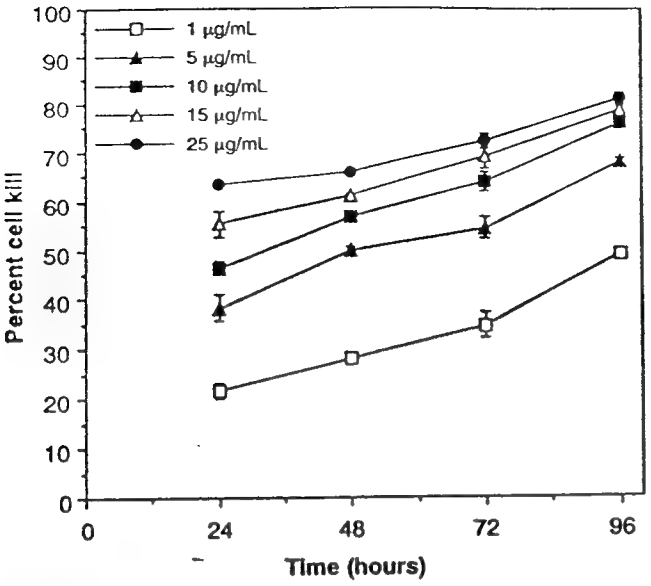

Figure 2. Dose–effect curves of merodantoin against MCF-7 cells. Cells (5×10^4 cells/ml) were treated with indicated doses of merodantoin. After incubation at 37°C for 24, 48, 72, or 96 hours, the cell viability was determined by methylthiotetrazole assay. Results (mean ± standard error of the mean) of three separate experiments performed in quadruplicate are shown.

rapid. A 96-hour treatment of cells with 1 μg/ml of merodantoin caused a 49.0 plus or minus 0.72% cell kill, whereas at a dose of 25 μg/ml, the cell kill increased to 81.3 plus or minus 1.2%. Tumor cell kill also increased from 64% to 82% when cells were incubated with 25 μg/ml of merodantoin for 24 hours and 96 hours, respectively.

Effect of pMC540 on Inhibition of Clonogenic Growth

In another set of experiments (Table 1), MCF-7 cells were treated with different doses of pMC540 or merodantoin for 48 hours and then trypsinized, washed, and plated in clonogenic assays. Results show that a 40 μg/ml dose of pMC540 and a 10 μg/ml dose of merodantoin caused a virtually complete inhibition of clonogenic growth.

Effect of pMC540 Injected Into Established Breast Tumor Xenografts

In the first set of in vivo experiments, the effects of the intratumor injections of pMC540 were investigated. Athymic mice with MCF-7 cells growing as solid tumors received intratumor injections of pMC540 (250 mg/kg) on alternate days after the tumor reached an average area of 3.3 cm^2. The area of the implanted tumors was 1.9 cm^2. Results show that the growth of tumors treated with eight doses of pMC540 (250 mg/kg) over a period of 16 days was significantly inhibited ($P < 0.001$) (Table 2). All treated tumors had regressed 16 days after the initiation of injections, and tumor could not be detected in three of the animals. Untreated tumors continued to grow and reached an average area of 10.5 cm^2 at the end of 16 days.

Effect of pMC540 and Tamoxifen on the Growth of Breast Tumor Xenografts Mimicking the Early Stages of Tumor Growth

In the next set of experiments, the effects of pMC540 injected intramuscularly were determined. The tumor growth curves for control and treated groups are shown in Figure 3. Data show that active growth of the xenografts (implantation size, 0.3 cm^2) continued for the duration of the experiments. Tamoxifen did not affect tumor growth rate. However, at the end of a 40-day (20-dose) treatment, the tumor growth was suppressed by 42% and 67% in pMC540 and pMC540 plus tamoxifen treated groups, respectively. An identical treatment of tumor-bearing animals with merodantoin caused a maximum inhibition (98%) of tumor growth compared with the controls (Fig. 3). The area, wet weights, and volumes of the tumors were compared. Results (Table 3) show that treatment of athymic mice bearing human breast xenografts with pMC540 (250 mg/kg) resulted in a significant ($P = 0.004$; $P = 0.0882$; $P = 0.0903$) sup-

Table 1. Effect of Preactivated Merocyanine 540 and Merodantoin on Clonogenic Growth of MCF-7 Breast Cancer Cells

Treatment	Clonogenic growth in MCF-7 cells (no. of colonies)
Untreated	204
pMC540 (80 μg/ml for 24 hr)	87
pMC540 (40 μg/ml for 48 hr)	0
pMC540 (80 μg/ml for 48 hr)	0
MD (10 μg/ml for 24 hr)	3
MD (10 μg/ml for 48 hr)	0
MD (15 μg/ml for 45 hr)	0

pMC540: preactivated merocyanine 540 (all treatments were made with preactivated merocyanine 540); MD: merodantoin

MCF-7 cells (5×10^5 cells/ml) were treated with pMC540 (40 μg/ml and 80 g/ml) or MD (5 μg/ml, 10 μg/ml, and 15 μg/ml) for 24 to 48 hr. After the incubation period, cells were plated in clonogenic growth medium. To prevent the attachment of cells to the plate, a layer of 3% methylcellulose was placed in the bottom of the wells. Colonies consisting of 50 or more cells were counted on day 7. Latent growth was monitored for 15 days. Results of a representative experiment carried out in triplicate are shown

Table 2. Effect of Intratumor Injections of pMC540 on MCF-7 Breast Tumor Transplanted Into Athymic Mice

Treatment group (n = 7)	Weights of animals (g) (mean ± SEM)	Drug dose (mg/kg)	Tumor area (cm^2) (on last day)	Tumor weight (g) (mean ± SEM)	Tumor volume (cm^3) (mean ± SEM)	% TGI
Control	23.1 ± 0.447	—	10.460 ± 1.978	0.719 ± 0.1632	0.4981 ± 0.166	
pMC540*	23.9 ± 0.786	250	2.120 ± 0.770 ($P = 0.0026$)	0.2645 ± 0.042 ($P = 0.0056$)	0.068 ± 0.029 ($P = 0.00256$)	63.21

Route of drug administration: intratumor, 8 doses on alternate days; size of tumor implanted: 5 × 5 mm; treatment initiated when tumor size was 7 × 6 mm.
% TGI indicates percent tumor growth inhibition, calculated by using the formula % TGI = $100(1 - W_t/W_c)$, where W_t and W_c are the mean weights of the treated and control tumors, respectively.

pression in tumor area, but only a marginally significant suppression of tumor weight and volume, respectively. Overall, there was a 43% suppression compared with the untreated controls. In the group of animals receiving a combination of tamoxifen and pMC540, the reduction in tumor area, weight, and volume was highly significant ($P = 0.0002$; $P = 0.029$; $P = 0.0069$, respectively), although the dose of pMC540 used was reduced from 250 mg/kg to 100 mg/kg.

Effect of Merodantoin on the Growth of Breast Tumor Xenografts

Another group of animals bearing human breast tumor was treated with merodantoin, a chemically synthesized photoproduct present in pMC540. The effect of merodantoin on MCF-7 growth 40 days after the start of the experiment is shown in Table 4. In two of the six animals, the implanted tumor was not detectable at the time of assessment. In the remaining four animals, the tumors did not grow beyond the implantation size of 0.3 cm^2. Photographs of representative animals from this group are shown in Figure 4. Compared with the results with pMC540, merodantoin may have greater antitumor activity (Fig. 3).

Discussion

In previous studies, we have reported that the novel approach of preactivation, when applied to the inactive photoactive compounds, results in the formation of heretofore unknown compounds that exert their biologic effects independent of light.[7–12] We also have reported that pMC540 preferentially kills certain types of tumors, while normal cells and tissues are left virtually unaffected.[7,10] However, the effects of these compounds against breast cancer cells in vitro and breast tumors in vivo have not been investigated. In the current study, we attempted to determine whether pMC540 and/or merodantoin are effective in inhibiting the growth of MCF-7 cells in vitro as well as xenografted MCF-7 breast tumors in athymic mice.

Treatment of MCF-7 cells in vitro with pMC540 and merodantoin produced a dose- and time-dependent cytotoxicity. Cytotoxicity at 24 hours appeared to be greater with merodantoin than pMC540; however,

Table 3. Effect of pMC540, Tamoxifen, and Tamoxifen + pMC540 on the Growth of MCF-7 Tumor

Treatment group (n = 10)	Weights of animals (g) (mean ± SEM)	Drug dose	Tumor area (cm^2) (mean ± SEM)	Tumor weight (g) (mean ± SEM)	Tumor volume (cm^3) (mean ± SEM)	% TGI
Control*	22.2 ± 1.31	Vehicle only	10.780 ± 1.44	0.797 ± 0.141	0.800 ± 0.141	
pMC540	19.1 ± 2.92 ($P = 0.3529$)	250 mg/kg	5.030 ± 1.02 ($P = 0.0044$)	0.455 ± 0.123 ($P = 0.882$)	0.471 ± 0.118 ($P = 0.903$)	42.91
Tamoxifen	23.8 ± 2.29	5-mg pellet mouse	11.090 ± 1.700 ($P = 0.8909$)	1.059 ± 0.177 ($P = 0.2604$)	1.058 ± 0.172 ($P = 0.2609$)	
Tamoxifen + pMC540	23.9 ± 2.53	5-mg pellet + 100 mg/kg	3.310 ± 0.710 ($P = 0.0002$)	0.263 ± 0.071 ($P = 0.029$)	0.311 ± 0.077 ($P = 0.0069$)	67.0

% TGI was calculated as described in Table 2.
* Implantation size approximately 0.2 cm^2. Treatments with pMC540 were given on alternate days via intramuscular injections for 40 days. Final tumor size assessments were made on day 40.

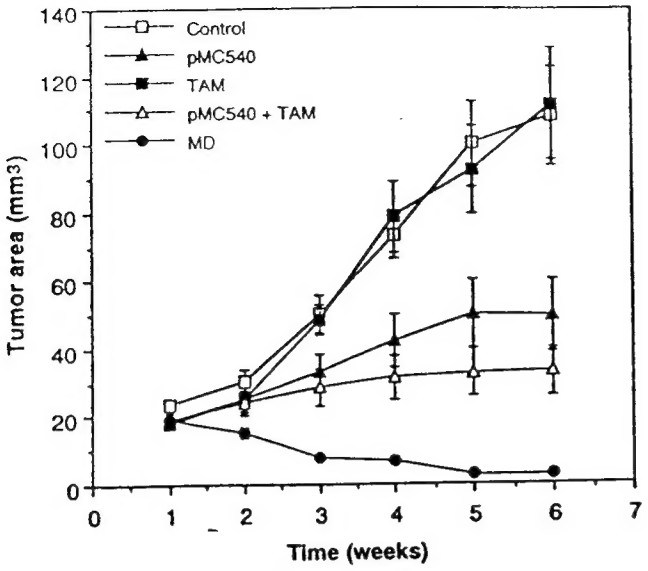

Figure 3. Tumor growth (mean ± standard error of the mean) curves for MCF-7 xenografts in the presence of continuous estrogen (control) and treated with pMC540 (250 mg/kg), tamoxifen (TAM) (5 mg pellet), TAM plus pMC540 (5 mg pellet + 100 mg/kg), and merodantoin (MD) (75 mg/kg). Alternate-day treatment of mice was initiated 24 hours after tumor implantation. There were 10 mice per group. The standard error of the mean for MD ranged from 1.0 to 1.6 mm, too small to be seen on the graph.

both agents were effective in killing MCF-7 breast cancer cells in vitro. Direct injection of pMC540 into tumors allowed exposure of the tumors to the unmetabolized form of the experimental compound. Results presented here demonstrate that only a brief treatment (eight doses) with intratumor injections of pMC540 was effective in causing a dramatic regression of breast tumors. Forty-three percent of the treated animals did not show palpable tumors at the end of an eight-dose, 16-day treatment. In the remaining animals, there was a significant (P = 0.002) regression (63%) of the implanted tumors, suggesting that pMC540 is an effective antitumor agent under in vivo conditions.

Encouraged by these results, the effects of intramuscularly injected pMC540 on small tumors (2 × 2 mm) mimicking the early stages of growth were determined. In the presence of estrogen pellets, the doubling of tumor size in the control group was approximately 10 days, which is in agreement with the previous reports.[20] In our experiments, the treatment of animals with pMC540 caused a significant ($P < 0.001$) suppression of tumor growth as determined by tumor area, weight, and volume. Consistent with previously published reports, tumor weight correlated best with tumor volume determined by pyknometry.[20] Treatment with tamoxifen alone did not produce an observable inhibition of growth under the conditions used. This may be due to the higher affinity of breast cancer cells for estrogen than for tamoxifen. The growth rate of MCF-7 xenografts is dependent on estradiol concentration.[16,17] It has been reported that administration of tamoxifen to rapidly growing, estrogen-stimulated xenografts results in a dose-dependent retardation of growth.[21–24] The differences between our study and the published reports are probably due to the concentration of estradiol and tamoxifen used as well as the duration of the treatment. Several studies have used 0.5–1.0 mg of estradiol, which can produce serum levels of 300–600 pg/ml. It has been reported that at these levels of estrogen, stimulated MCF-7 tumor growth can be inhibited by tamoxifen producing serum levels of 40–50 ng/ml.[25] However, in our study, the doses of estrogen and tamoxifen used were 1.7 mg and 5 mg, respectively. These doses have been reported to produce serum levels above 900 pg/ml and 4–5 ng/ml, respectively.[26] Thus the observed lack of growth inhibition by tamoxifen alone may be due to the concentrations of estrogen and antiestrogen used and not due to the inactivity of tamoxifen. It has been reported that growth inhibitory effects of tamoxifen can be partially reversed if serum estradiol concentration is greater than 900 pg/ml.[25] These serum levels mimic the increase often observed clinically in premenopausal women who are taking tamoxifen. In a

Table 4. Effect of Merodantoin on the Growth of MCF-7 Tumor

Treatment group	Weights of animals (g) (mean ± SEM)	Drug dose (mg/kg)	Tumor area (cm^2) (mean ± SEM)	Tumor weight (g) (mean ± SEM)	Tumor volume (cm^3) (mean ± SEM)	% TGI
Control* (n = 10)	24.1 ± 1.31	Vehicle only	10.780 ± 1.44	0.797 ± 0.141	0.800 ± 0.141	
Merodantoin (n = 6)	25.3 ± 0.56	75	0.211 ± 0.104 (P = 0.0001)	0.011 ± 0.004 (P = 0.0009)	ND	98.62

TS: too small for accurate measurements.

% TGI was calculated as described in Table 2.

* Implantation size, approximately 0.2 cm^2. Treatments with merodantoin were given on alternate days via intramuscular injections for 40 days. Final tumor size measurements were made on day 40.

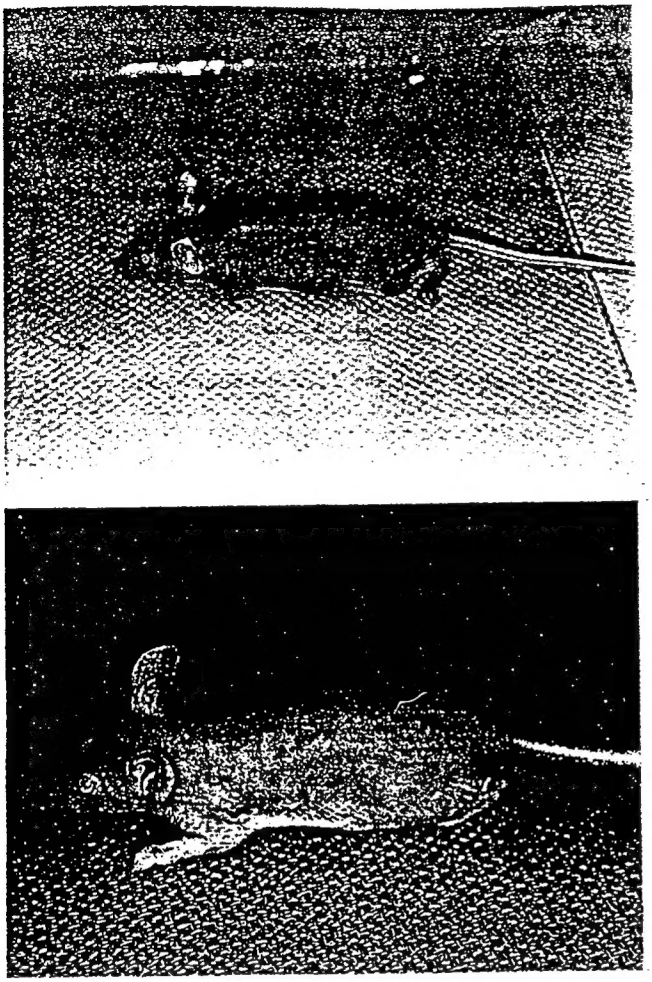

Figure 4. Photographs of athymic mice bearing human breast tumor. (Top) Control, treated with vehicle only; (bottom) treated with merodantoin (75 kg/mg) on alternate days for 40 days.

recent study, Brunner et al. found no difference in the theoretical clonogenic doubling time (assuming no cell loss) of tamoxifen-treated tumors.[27]

Nonetheless, a combination of tamoxifen and pMC540 produced a more pronounced inhibition of growth. These findings may be useful for reducing the therapeutic doses of both agents. It is also interesting to consider that tamoxifen has been shown to antagonize the cytotoxic effects of several chemotherapeutic agents, such as melphalan and fluorouracil in vitro.[28,29] Combination therapy frequently is used for the treatment of breast cancer. Whether pMC540 will affect this antagonism remains to be determined. The mechanism of this cooperation between tamoxifen and pMC540 is not clear and is the subject of a separate study. It will be of interest to determine whether pMC540 has an effect on the number or affinity of receptors on breast cancer cells for tamoxifen and/or estrogen. It is not clear at this time whether the higher degree of tumor growth inhibition observed with merodantoin is simply due to a potentially longer plasma half-life, higher plasma concentration, or other factors just described.

One mechanism of action of pMC540 and merodantoin appears to involve intracellular induction of reactive oxygen species,[11] leading to alterations in calcium homeostasis, DNA damage, and apoptosis (submitted for publication).

During the course of these experiments, it was observed that both pMC540 and merodantoin were easily tolerated without discernible differences in the general well-being of the treated and control groups. Weight loss in any of the treatment groups was not observed. A slight nonsignificant increase in weight of treated groups may be attributable to better food intake due to reduced or, in some cases, lack of tumor burden. Gross examination at autopsy of tumor-bearing animals in the treated groups did not reveal a noticeable change in lungs, liver, spleen, or kidneys compared with the control animals, suggesting that these compounds were easily tolerated at the doses used. For pMC540, these observations are consistent with our previous experience.[9,11,12] These results suggest that compounds generated through the process of preactivation may be useful as therapeutic agents. Studies are under way to determine the effects of these novel compounds in the presence and absence of hormonal manipulation on growth of established breast tumors.

Conclusion

pMC540 and its purified photoproduct merodantoin were effective in suppressing the growth of human MCF-7 tumors xenografted into athymic mice. These compounds were also effective in the presence of tamoxifen.

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